Vol. XLII, No. 5 (October, 1961), was issued on 18.10.61.

THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XLII DECEMBER, 1961 NO. 6

INVESTIGATIONS ON AUTOTRANSPLANTATION OF THE ADRENAL GLAND INTO THE SPLEEN WITH SPECIAL REFERENCE TO PITUITARY-ADRENAL RELATIONSHIPS

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Received for publication May 12, 1961

THE existing methods for the cytochemical localization of the various pituitary hormones are not specific. These hormones do not possess specific distinguishing properties which could be exploited with advantage for the development of specific staining methods. However, data gathered from pituitary hyperfunction or depletion and changes in the target organs have thrown some light on the cellular source of some, if not all, of the hormones. Thus the growth and lactogenic hormones are supposed to be elaborated by the acidophil cells, and the source of the two gonadotrophic hormones and the thyrotrophic hormone is traced to the basophil cells. However, there appears no unanimity of opinion regarding the cytological source of adrenocorticotrophic hormone (ACTH). The Crooke-Russell cells seen in the pituitary glands of patients suffering from Addison's disease, Crooke's hyaline basophil cells seen in patients suffering from Cushing's syndrome and the amphophils have at various times been associated with the production of ACTH (Everson Pearse, 1953 ; Russfield, 1955).

In any attempt to localize the cellular source of adrenocorticotrophic hormone, it was necessary first to stimulate the pituitary gland to secrete increasing quantities of ACTH. Sydnor and Sayers (1954) and Fortier (1958) reported an increase both in the production and secretion of ACTH following bilateral adrenalectomy and prolonged reduction of corticoid levels in circulation. However, corresponding morphological changes in the pituitary gland have not been reported. It was therefore decided to autotransplant an adrenal gland into the spleen immediately following bilateral adrenalectomy, and study the pituitary cytology at various intervals of time. The rationale for following this procedure was as follows : (i) the regeneration of the adrenal autograft and its ability to synthesize corticoids could be taken as an index of ACTH production and secretion, and (ii) the corticoids produced by the transplanted adrenal would drain

directly into the liver where they would be rendered physiologically inactive by conjugation. The response in such animals was therefore expected to be similar to that seen in bilaterally adrenalectomized animals.

MATERIAL AND METHODS

Wistar rats of both sexes and of about 8–10 weeks of age were used for this study. They were maintained on stock diet and water *ad libitum*.

Animals were divided into a number of groups and were subjected to the following experimental procedures: (1) bilateral adrenalectomy alone, (2) bilateral adrenalectomy and autotransplantation of an entire adrenal gland under the capsule of the spleen, (3) bilateral adrenalectomy and autotransplantation of fragments of the adrenal gland into the mass of the spleen, (4) enucleation, (5) bilateral adrenalectomy and autotransplantation of fragments of the adrenal gland into the spleen, followed by corticosterone treatment, and (6) bilateral adrenalectomy and transplantation of abdominal muscle into the spleen. (7) A group of normal animals served as controls.

The animals were killed at various intervals of time following the operative procedures. Liver, kidney, spleen bearing the adrenal graft, thyroid, ovary or testis and accessory sex glands removed at the time of autopsy were fixed in Bouin's fluid. Paraffin sections of the tissues, cut at a thicknes of 5 microns, were stained with Ehrlich's haematoxylin and aqueous eosin and also with picro-Mallory for the demonstration of connective tissue.

Pituitary gland was fixed in Zenker-formol. Paraffin sections of the gland were stained with picro-Mallory and with the Trichrome-PAS method of Everson Pearse (1949), with the modification that aurantia in phosphotungstic acid was substituted for Orange G to stain acidophils.

RESULTS

The mortality rate was high in the operated animals (80 per cent). Adhesions and vascular communications between the adrenal graft in the spleen and the systemic circulation were present in all animals in which an entire adrenal gland was autotransplanted under the capsule of the spleen. In animals in which fragments of adrenal tissue were transplanted into the mass of the spleen, there were no vascular collaterals, although adhesions were present.

Group 2: Bilateral adrenalectomy and autotransplantation of an entire adrenal gland under the capsule of the spleen

The first batch of animals from this group was killed 10 days following the operative procedure and the last batch of animals two months later.

Ten to fifteen days following transplantation, the grafted adrenal tissue was completely necrosed. Viable adrenocortical cells were not present. Plently of inflammatory cells and a few histiocytes were seen (Fig. 1). Three weeks later, regeneration was evidenced by the presence of new adrenocortical cells. Cytoplasm of these cells stained uniformly with eosin. Nuclei were vesicular (Fig. 2). In about two months there was considerable regeneration of the graft. The graft had differentiated into a 4–6 cell thick zona glomerulosa and a broad fasciculata. There was no further differentiation of the regenerated cortex into a zona reticularis (Fig. 3). Adrenal medullary tissue was not present in any of the transplanted glands.

Pituitary gland:—Microscopic examination of the pituitary gland revealed no marked alterations in the morphology or population of the three cell types. There were, however, a cluster of small hyperchromatic cells in the adenohypophysis. Cytoplasm of these cells stained variably with aniline blue and

also with PAS (Fig. 4). Such cells were not present either in the adenohypophysis of the normal rats from Group 7 (Fig. 5) or rats subjected to bilateral adrenalectomy alone from Group 1 (Fig. 6).

Group 3 : *Bilateral adrenalectomy and autotransplantation of fragments of the adrenal gland into the mass of the spleen*

The shortest time between operation and death in this group of animals was 8 days and the longest was 3 months. The fragments of the adrenal gland were able to establish vascular communications with the spleen in a comparatively short duration of time, with the result the regenerative process was considerably accelerated.

A week following autotransplantation, the transplanted adrenal fragment was completely necrosed. Degenerating cells, inflammatory cells and foreign body giant cells were present at the site of the graft (Figs. 7, 8 and 9). Two weeks later, encapsulated islands of regenerated cortical cells were present, although the necrotic area still persisted (Fig. 10). Three or four weeks later there was an increase in the number of regenerated cells. Mitotic figures were also noted (Fig. 11). By 4 weeks, the regenerated cells in the graft had differentiated into 2 distinct zones, the zonae glomerulosa and fasciculata. Cytoplasm of these cells was finely vacuolated and in frozen sections it stained positive after the ferric chloride Schiff's (FCS) method for corticoids (Figs. 12 and 13). The cortex had restituted more or less completely by 10 weeks. Adrenal medullary tissue was not present.

Pituitary gland.—Grossly the pituitary gland appeared normal. On microscopic examination, there appeared to be a moderate increase in the number of mucoid basophil cells. A characteristic feature was the presence of randomly distributed clusters of small epithelial cells in the anterior lobe. These cells measured about 7–9 microns in diameter. Cytoplasm of these cells stained positive with aniline blue and also after PAS. The negative Golgi image was not distinct. Nuclei were hyperchromatic (Figs. 14 and 15). These cells persisted even 10 weeks after transplantation. The intermediate and neural lobes were normal.

Group 4 : *Enucleation*

Enucleation was carried out in a group of animals to study the regeneration *in situ* of the adrenal gland from the capsular elements, keeping the anatomical site and blood supply intact and also to correlate the pituitary cytology with adrenal regeneration.

In animals killed a week after enucleation, the central area (which contained the cortex and medulla before enucleation) was full of cellular debris and inflammatory cells (Fig. 16). A pronounced cellular activity in the capsule was evident. Regenerated cortical cells were present and they were arranged in the form of plates or cords. Cytoplasm of these cells was either homogeneous or vacuolated. Nuclei were vesicular (Fig. 17). Three or four weeks later there was more or less a complete restitution of the cortex. The cortex had differentiated into a zona glomerulosa and a zona fasciculata (Fig. 18).

Pituitary gland.—In the pituitary glands of these animals, there were clusters of small hyperchromatic PAS-positive basophil cells (Fig. 19). The other types of cells appeared normal.

Group 5 : Bilateral adrenalectomy and transplantation of adrenal fragments into the spleen, followed by corticosterone treatment

In this group of animals, a fragment of the adrenal tissue was transplanted into the spleen following bilateral adrenalectomy. Fifteen days later, corticosterone at a dose of 1 mg./ml. in propylene glycol was injected intramuscularly every alternate day for 12 days. The total amount of corticosterone so injected was 6 mg. The animals were killed 24 hr. following the last injection. An equivalent amount of propylene glycol was injected into three normal rats, and these were killed along with the other experimental animals.

EXPLANATION OF PLATES

FIG. 1.—Grafted adrenal 2 weeks after autotransplantation of an entire adrenal gland under the capsule of the spleen. Note the inflammatory cells. H. and E. ×108.

FIG. 2.—Graft 4 weeks after transplantation of an entire adrenal gland into the spleen; the cortical cells are regenerating from the periphery and there is a small area of necrosis in the centre. H. and E. ×108.

FIG. 3.—Regenerated cortex 2 months after transplantation of an entire adrenal gland into the spleen. Only the glomerulosal and fasciculate zones are distinct. Note the cytoplasmic vacuolization. H. and E. ×108.

FIG. 4.—Anterior hypophysis of a rat bearing a regenerated cortex in the spleen. Note the small hyperchromatic cells. PAS. ×166.

FIG. 5.—Anterior hypophysis of a normal rat. PAS. ×166.

FIG. 6.—Anterior hypophysis of an adrenalectomized rat. PAS. ×166.

FIG. 7.—Degeneration of the adrenal fragment in the spleen 8 days after transplantation. H. and E. ×23.

FIG. 8.—High power view of Fig. 7. Note the marked invasion of inflammatory cells. H. and E. ×108.

FIG. 9.—Section from the spleen of another animal with a degenerated adrenal fragment to show a foreign body giant cell. H. and E. ×433.

FIG. 10.—An island of regenerating cortical cells, 2 weeks following transplantation of an adrenal fragment into the spleen. A small area of necrosis is indicated by an arrow. H. and E. ×75.

FIG. 11.—Mitotic figures in the regenerating cortex, 3 weeks after transplantation of an adrenal fragment into the spleen. Mallory. ×332.

FIG. 12.—Differentiation of the regenerated cortex into 2 zones, 6 weeks after transplantation of an adrenal fragment into the spleen. H. and E. ×75.

FIG. 13.—Presence of a corticoid-like material in the regenerated cortical cells 3 weeks after transplantation of an adrenal fragment into the spleen. Aniline FCS. ×108.

FIG. 14.—Anterior hypophysis of a rat with a regenerating adrenal cortex in the spleen. Note the clusters of small hyperchromatic PAS positive cells. PAS. ×166.

FIG. 15.—High power view of Fig. 14. PAS. ×332.

FIG. 16.—Inflammatory cells and necrosis 1 week after enucleation of the adrenal gland. H. and E. ×108.

FIG. 17.—Cortical cells regenerating from the periphery following enucleation. Note a large area of haemorrhage. Mallory. ×108.

FIG. 18.—Differentiation of the regenerated cortex a month after enucleation. H. and E. ×108.

FIG. 19.—Presence of small hyperchromatic PAS positive cells in the anterior hypophysis of a rat after enucleation. PAS. ×290.

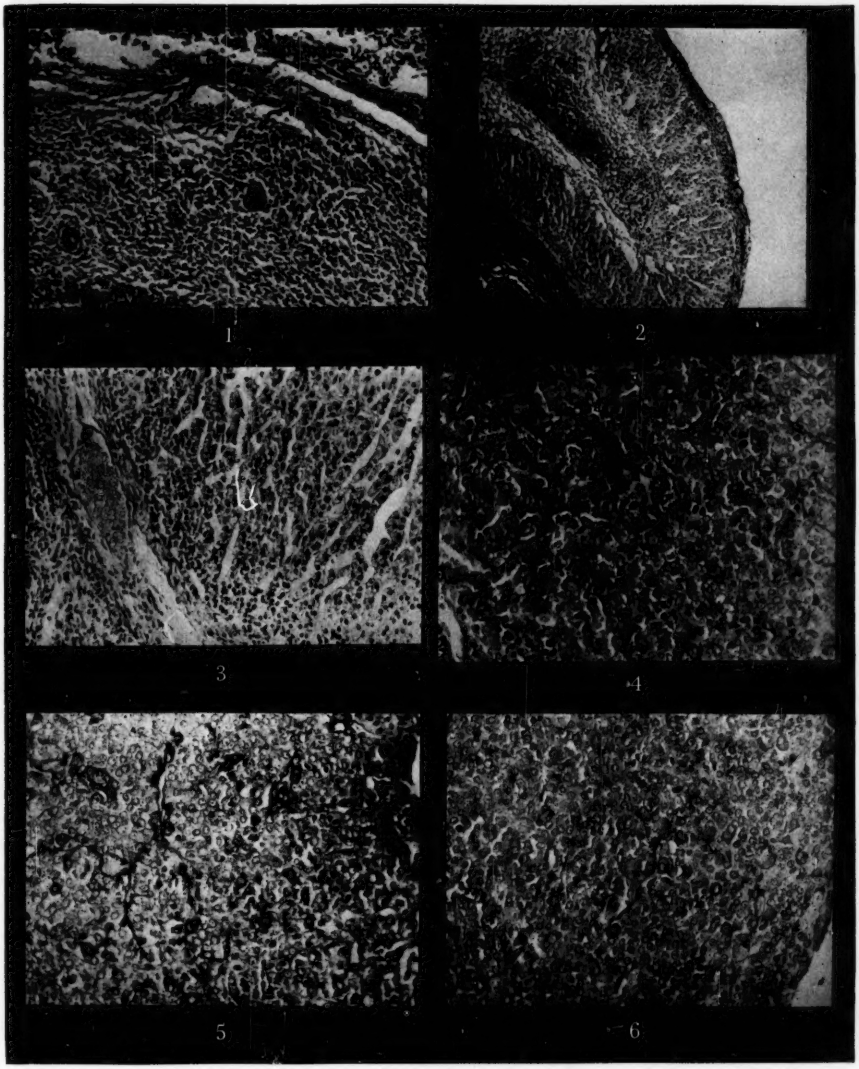
FIG. 20.—Transplanted adrenal tissue from a rat treated with corticosterone as described in text (Group 5). Note the presence of hyaline areas and vesicular nuclei. No viable adrenocortical cells are present. Mallory. ×216.

FIG. 21.—Anterior hypophysis of the same animal (described in Fig. 20). Note the presence of small cells with hyperchromatic nuclei. PAS. ×166.

FIG. 22.—A piece of abdominal muscle grafted into the spleen following bilateral adrenalectomy. Note the absence of muscle bundles, although fragments of muscle fibres are seen. Mallory. ×108.

FIG. 23.—Anterior hypophysis of the same animal described in Fig. 22. Note the absence of hyperchromatic cells. PAS. ×166.

FIG. 24.—Enlarged corpora lutea in the ovary of a rat with an intrasplenic adrenal graft. H. and E. ×24.



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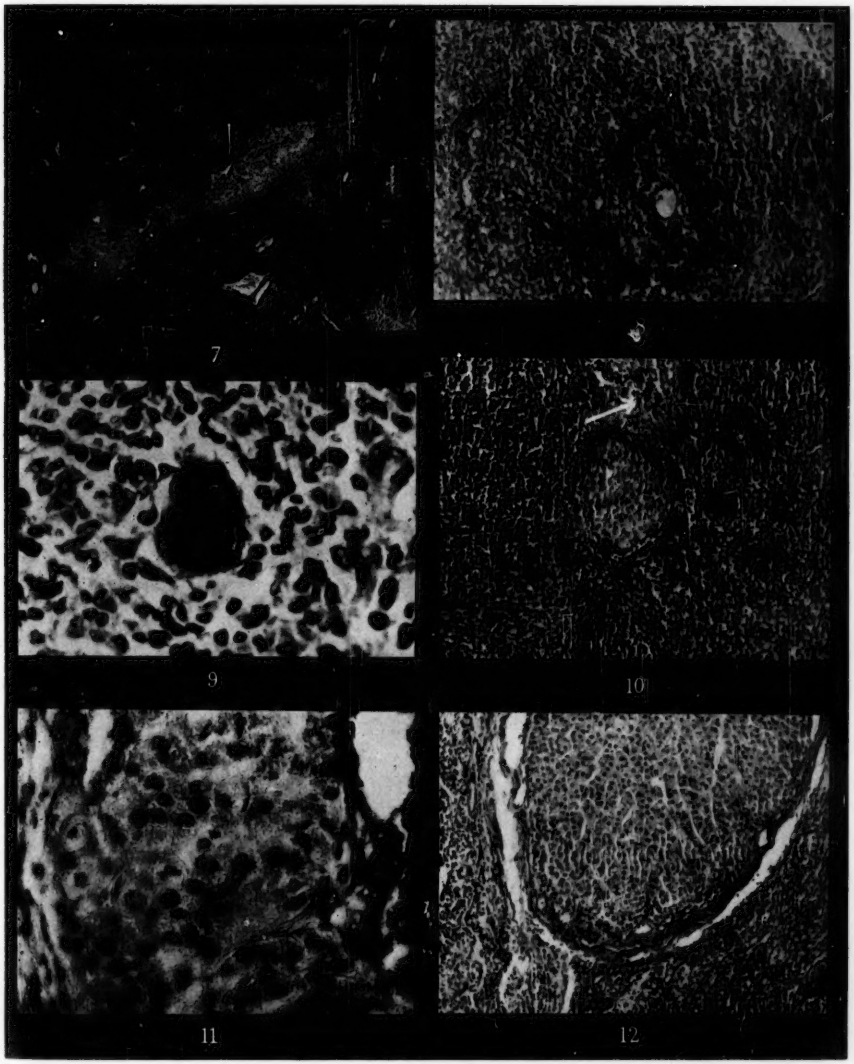

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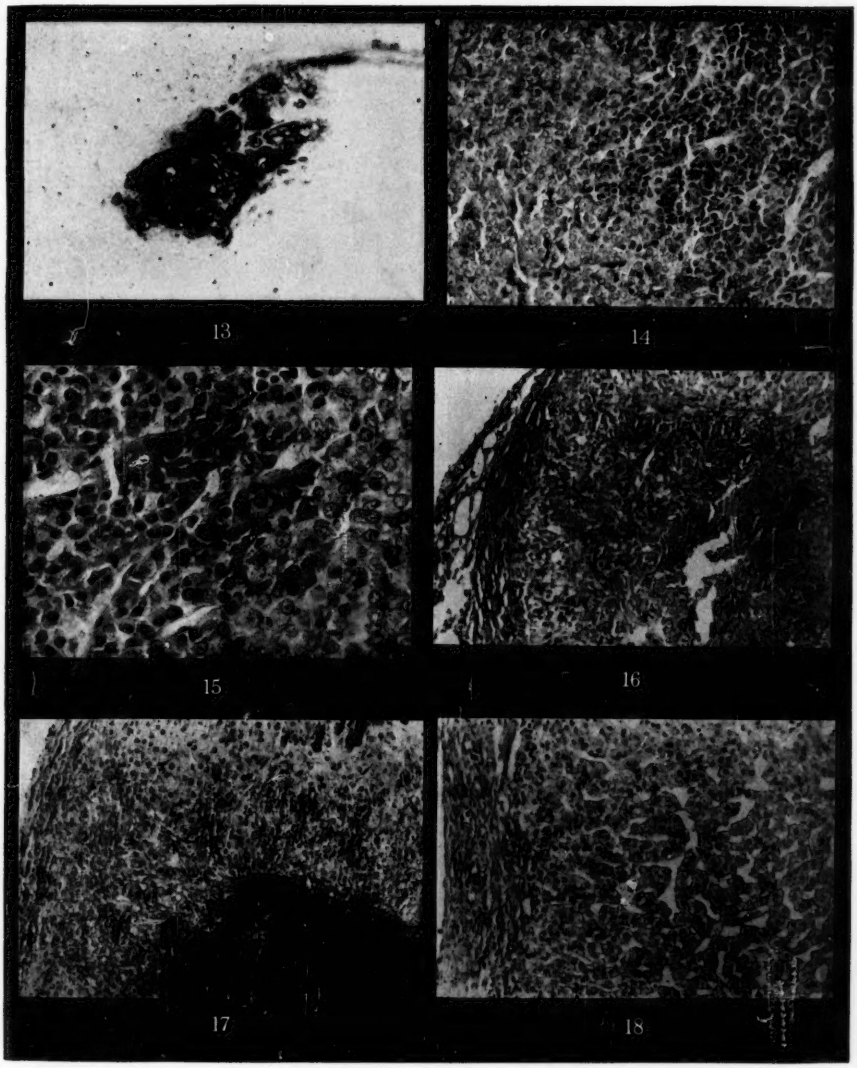

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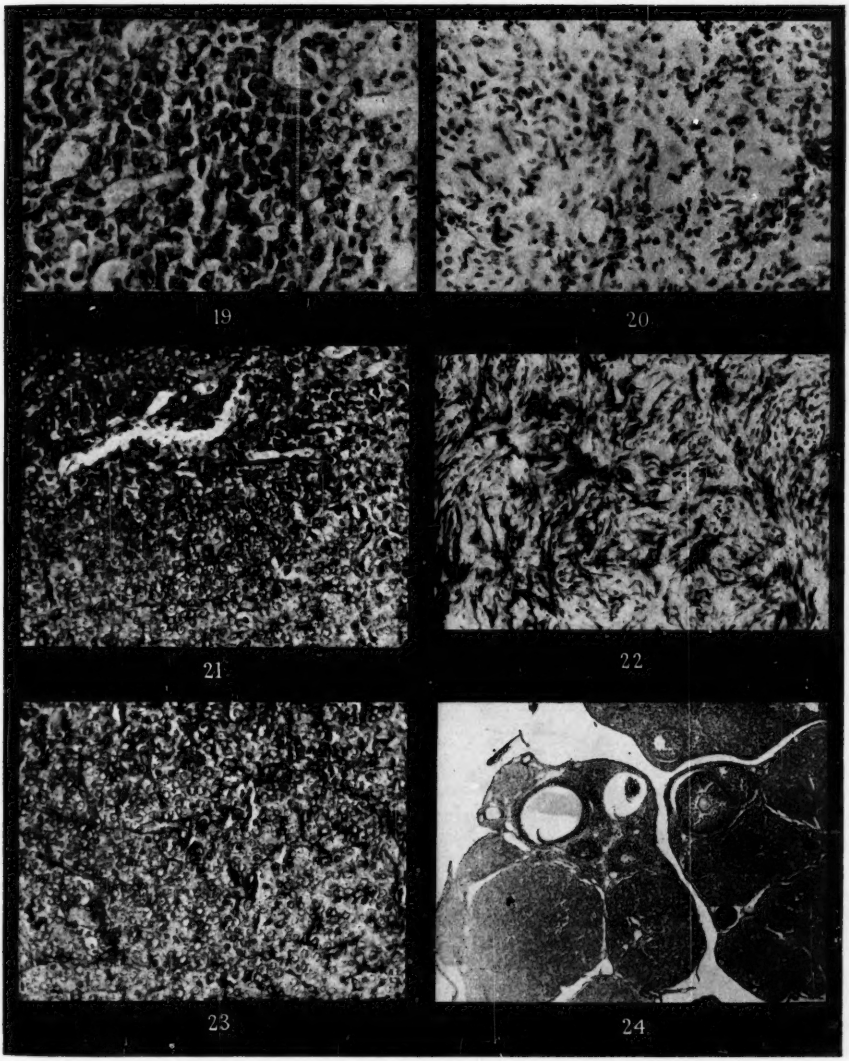

19 20

21 22

23 24

At the site of transplantation in the spleen of the experimental animals, adrenocortical tissue was not present. Instead, collagen fibres encapsulating islands of vesicular nuclei were present. There was no organization amongst these syncitia of nuclei. A few inflammatory cells were present (Fig. 20).

Pituitary gland.—Microscopic examination of the pituitary gland revealed a persistence of the hyperchromatic basophil cells (Fig. 21). However, they were rather few. The morphology and population of the other cell types remained unaltered.

Group 6 : *Bilateral adrenalectomy and transplantation of abdominal muscle into the spleen*

This experiment was carried out to study the response of the pituitary gland to non-specific trauma to the spleen. For this purpose a piece of abdominal muscle was introduced into the spleen of a group of bilaterally adrenalectomized rats. The animals were killed 1–3 weeks after the operative procedure.

The grafted muscle, transplanted into the spleen, was completely necrosed. Intact muscle bundles were not observed (Fig. 22). In the pituitary glands of such animals, hyperchromatic basophil cells were not present (Fig. 23).

Other endocrine glands.—Except for a slight degree of variation in the size of the follicles, the thyroid gland in the animals with an intrasplenic graft appeared normal. The height of the lining cells did not differ much from that seen in normal rats.

The corpora lutea in the ovaries of rats with an intrasplenic graft (Groups 2 and 3) were larger in size as compared with those of the normal animals (Fig. 24). In addition there appeared a moderate increase in the volume of the stromal cells.

DISCUSSION

The salient features of this study were : (1) The grafted adrenal tissue regenerated progressively into a fully developed and functional cortex, (2) No significant morphological changes in the thyroid gland and gonads of these animals were seen, (3) Clusters of small hyperchromatic PAS-positive basophils appeared in the anterior lobe of the pituitary gland whenever fragments of adrenal tissue were present in association with diminished levels of circulating corticoids.

The regenerated cortex differentiated into glomerulosa and fasciculata zones only. These observations are in contradistinction to those made by Ingle and Higgins (1938), Butcher (1948) and Skelton (1959), who reported the presence of all the three zones. The cells in the regenerated cortex appeared to be functionally active. There was marked vacuolization in the cytoplasm of the regenerated cortical cells and the cytoplasm (in frozen sections) stained positive with sudan dyes and also after the ferric chloride Schiff's (FCS) method (Khanolkar, Krishna Murthy, Bagul and Sahasrabudhe, 1958), thereby indicating the presence of steroids with an alpha ketol group. This positive staining indicated that the regenerated cortex was capable of producing and secreting corticoids. This was further substantiated by the fact that saline supplementation was not necessary eight or ten days after transplantation (when the graft became functional), and the glycogen picture in the livers of these animals was normal.

In these investigations hyperchromatic PAS positive basophil cells appeared in the anterior hypophysis concomitant with the regeneration of the transplanted adrenal tissue. This close parallelism suggests that these cells may be responsible for the elaboration of adrenocorticotrophic hormone. Since basophils have also been associated with the elaboration of the thyrotrophic and the two gonadotrophic hormones, the morphology of the thyroid gland, the ovary and testis was also studied to investigate whether the new types of cells also secreted these hormones. The observations suggest that there was no overt stimulation of these tropic hormones.

Regeneration of the adrenal cortex either following enucleation or after transplantation is dependent upon the pituitary adrenotrophic principle. The regeneration of the adrenal graft, its differentiation and its secretory capacities can only be attributed to the increased secretion of pituitary ACTH. Administration of exogenous corticosterone (which was resorted to in Group 5 to inhibit the pituitary from secreting ACTH) to the animals with an intrasplenic graft resulted in an atrophy of the regenerated cortex.

In the pituitary glands of the enucleated animals and those with an intrasplenic graft, there were small hyperchromatic PAS-positive cells in the adenohypophysis. Apparently a diminished level of circulating corticoids is not the only requisite for the appearance of these cells, because such cells were not present in animals subjected to bilateral adrenalectomy alone. The presence of the target cells appears to be absolutely necessary for the appearance of these cells.

In one group of animals in which an entire adrenal gland was transplanted into the spleen, there were plenty of adhesions and vascular communications between the grafted tissue and the systemic circulation. Hyperchromatic cells in the anterior lobe of these animals were very few. In these animals, perhaps, the corticosteroids secreted by the adrenal graft drained directly into the systemic circulation thereby exerting a regulatory influence on the pituitary gland. In animals with an adrenal graft well within the substance of the spleen, the corticosteroids had to pass through the liver, where they are made physiologically inactive by conjugation. (It is presumed that the conjugated corticoids are ineffective in inhibiting the secretion of ACTH). This might account for an increase in the number of PAS positive cells in the adenohypophysis. A close parallelism is worth noting between the present studies with an intrasplenic adrenal graft and the observations of Greep and Chester Jones (1950) on the intrasplenic ovarian graft.

In the pituitary glands of animals treated with corticosterone, the hyperchromatic cells were seen but they were very few. Further these cells did not appear in the pituitary glands of animals in which a piece of abdominal muscle was transplanted into the spleen following bilateral adrenalectomy.

SUMMARY

Based on these data the following conclusions may be justifiable. (1) Hyperchromatic PAS positive basophil cells which appear in the anterior lobe of the pituitary gland during adrenal regeneration may be responsible for the secretion of adrenocorticotrophic hormone. (2) These cells do not secrete the thyrotrophic hormone or the gonadotrophic hormones in appreciable quantities. (3) These cells appear in response to depletion of circulating corticoids, and the presence of

target organs is essential for their appearance. (4) Non-specific trauma (implantation of muscle in spleen) does not result in the appearance of these cells, although the levels of circulating corticoids was diminished.

This work was aided by grants from the Indian Council of Medical Research. This report forms part of a thesis submitted by one of us (A.S.K.) for the degree of Doctor of Philosophy of the University of Bombay.

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EXPERIMENTAL AUTO-IMMUNE THYROIDITIS IN THE RAT

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Received for publication May 13, 1961

THE production of inflammatory lesions in tissues containing organ-specific antigens by means of auto- or iso-immunization is now well founded and examples may be drawn from such diverse tissues as the brain, peripheral nerve, testes, thyroid and adrenal (see Waksman, 1959). These studies have a particular relevance to a number of human diseases suspected of having an auto-immune aetiology. In these diseases circulating auto-antibodies are found and the pathological lesions seen are of an inflammatory nature, in many ways comparable with those induced by experimental auto-immunization.

In the human, Hashimoto's thyroiditis, primary adult myxoedema, and the focal thyroiditis commonly encountered in Graves' disease, are frequently associated with high levels of circulating thyroid auto-antibodies and detailed studies have been made of the immune systems involved (Roitt and Doniach, 1960). Histologically the human glands most commonly show a marked infiltration by small lymphocytes, large mononuclear and plasma cells; this may be focal in nature or may extend throughout the whole gland and replace the normal thyroid architecture in extreme cases. Acinar breakdown with leakage of colloid, eosinophilic metaplasia of the epithelial cells, lymphoid germinal follicles and fibrosis may be observed, all to varying degrees. While it is not yet possible to decide whether the establishment of thyroid auto-immunity is responsible for the development and progression of these lesions or is merely a non-pathogenic consequence of tissue destruction, Witebsky, Rose, Terplan, Paine and Egan (1957) have clearly shown that the normal thyroid is susceptible to invasive attack by lymphoid elements following auto-immunization and that the resulting lesions have many features in common with those present in the human gland. In these studies, rabbits were injected intra-dermally into the footpads with homologous thyroid extracts incorporated into the aqueous phase of a complete Freund adjuvant; circulating thyroid antibodies and thyroiditis of variable severity were observed within 2–5 months in a large proportion of the animals. Thyroiditis could also be induced in guinea-pigs and dogs. The resulting lesions have been described in detail by Terplan, Witebsky, Rose, Paine and Egan (1960) and in many respects resemble those seen in the Hashimoto gland.

If experimental auto-immune thyroiditis is to be studied as a possible model for the human disease, it would seem desirable to have a procedure which gives an adequately predictable histological response within a short time period. Preferably one should use an animal species of relatively uniform genetic constitution, which can be conveniently handled and accommodated in large numbers. This paper describes the consistent production of marked thyroiditis in the rat within

2–4 weeks of injection of homologous and heterologous thyroid extracts or thyroglobulin. Ultimate regression of thyroiditis could be observed and this phenomenon may have been largely responsible for our early failures to observe pathological changes in the thyroids of immunized rats since in these experiments the glands were not examined until several months after the initial injection.

These results were briefly summarized in a recent review (Roitt and Doniach, 1960).

MATERIALS AND METHODS

Animals.—Rats were obtained from the Institute's own colony originally derived from the Wistar strain and maintained for about 15 yr. by a system of open breeding without inclusion of new stock. Male animals 2–3 months old were used for immunization.

Antigens.—Rat thyroids were stored at −20° until required. For the preparation of extracts, the glands or other organs were minced thoroughly with fine scissors and disrupted in a loose-fitting all-glass Potter-type homogenizer with 2 vol. of isotonic phosphate-buffered saline pH 7·2. The homogenate was spun at 2000 *g.* for 10 min. and the supernatant used for immunization. A preparation of rat thyroglobulin was obtained by taking the fraction of the thyroid extract which precipitated between 37 and 50 per cent saturation with ammonium sulphate; the precipitate was dialyzed and freeze-dried. In later experiments the thyroglobulin was sedimented by high speed centrifugation before salt fractionation. The products were homogeneous by the somewhat insensitive criterion of paper electrophoresis in barbitone buffer pH 8·6, $I = 0{\cdot}05$. Preparations of human and sheep thyroglobulin were obtained by the ammonium sulphate method of Derrien, Michele and Roche (1948); one of the sheep preparations was further fractionated on DEAE-cellulose as described by Ingbar, Askonas and Work (1959) but no appreciable alteration in antigenic potency could be observed.

Adjuvants.—The following adjuvant mixtures were used: (A) Stock solution of 8·5 vol. Bayol F (light paraffin oil; Esso Oil Co.): 1·5 vol. Arlacel A (emulsifying agent; Atlas Powder Co.); the aqueous antigen was emulsified with an equal vol. of stock solution. (B) Stock solution of 5 vol. Bayol F: 1 vol. Arlacel A: 4 vol. Falba (emulsifying agent; Pfaltz and Bauer); 3 vol. stock solution to 1 vol. aqueous phase.

The stock solution was homogenized with either heat killed human tubercle bacillus (1 mg./ml.), or with the acetone and isopropyl ether extracted bacillary residue (1 mg./ml.), described by Colover (1954), immediately before preparing the emulsion with thyroid extract or a solution of thyroglobulin. The aqueous phase was added in small volumes to the adjuvant and emulsified between additions by repeatedly drawing up into a syringe and discharging through a B.W.G. 26 hypodermic needle. The stability of the emulsion was tested by allowing a drop to fall onto the surface of chilled water where it should remain as a discrete globule for several hours.

Induction of experimental thyroiditis.—Animals were immunized by injecting 0·05 ml. of the emulsion intradermally into the tail near its base avoiding the main blood vessels. If a boosting injection was required, a further 0·05 ml. was placed in the tail at a separate site. The pretracheal muscles, thyroid and trachea were removed *en bloc* and fixed in formol saline for sectioning at 7 μ. Unless otherwise stated the sections were stained with haematoxylin and eosin.

Scoring of thyroiditis.—The degrees of thyroiditis defined in the results section were allotted arbitrary scores as follows: Negative = 0; + = 1; ++ = 2; +++ = 3; ++++ = 4. The arithmetic mean score for each group of animals was calculated. It appeared to be a reasonable reflection of the overall response and was helpful in comparing the effectiveness of different experimental treatments.

Hemi-thyroidectomy.—This was performed under ether anaesthesia and the thyroid approached through a ventral mid-line incision extending from the top of the sternum to the anterior border of the submaxillary gland. The salivary glands were separated and the pretracheal muscles divided by blunt dissection. One thyroid lobe was held by the adjacent connective tissue and separated from the trachea with a fine scalpel, avoiding the recurrent laryngeal nerve. The isthmus was cut in the mid-line and the lobe finally freed while the thyroid artery was clamped; bleeding from the artery tended to occur when it was released

but stopped before the skin incision was closed. Handling of the thyroid tissue itself was avoided since histological artefacts are easily produced. The second lobe of the thyroid was not disturbed and remained *in situ*.

RESULTS

Histology of the experimental thyroid lesion

Immunization of rats with homologous and heterologous thyroid antigens leads to the development of thyroiditis of varying grades of severity, the histological features of which are described below under the arbitrary headings of mild, definite and severe thyroiditis, and severe thyroiditis with goitre.

Mild thyroiditis (+).—Macroscopically the gland appears to be normal. In histological sections one frequently observes a microfollicular architecture coupled with dilatation of blood vessels in the capillary plexus and a tendency for leucocytes within these vessels to adhere to the endothelium. Since these features are often seen in the thyroids of rats treated with Freund's adjuvant alone or combined with control tissue homogenates, they are considered to be symptomatic of a more generalized tissue reaction to the adjuvant itself and are scored as negative. A thyroiditis is classified as mild when in addition there are foci of inflammatory cells (Fig. 2), sometimes perivenular (Fig. 7), in association with follicles showing colloid loss, increased epithelial cell height and rupture of the epithelium with streaming of lymphoid cells into the lumen. Lymphocytes and histiocytes are the main cell types constituting such foci, with the lymphocyte predominating in the perivenular lesion. Polymorphonuclear leucocytes and plasma cells are rarely present in lesions of this grade.

Definite thyroiditis (++).—Although the thyroids are normal in size and colour, on histological examination they show several inflammatory foci becoming confluent in one or both lobes of the gland (Fig. 3), but parts of the gland remain normal. Again lymphocytes and histiocytes are the predominant cell types in these lesions but polymorphonuclear cells are occasionally seen within the lymphocytic stroma (Figs. 8 and 9). Fibrosis is sometimes evident when the interfollicular spaces become densely infiltrated (Fig. 9). Trypan blue injected i.p. 24 hr. before killing is found within the cytoplasm of many of the histiocytes. Several follicles in the areas of inflammation may show degenerative changes: loss of colloid is marked and rupture of the epithelium with invasion of the lumen is common. Colloid resorption is not always associated with an apparent discontinuity in the epithelium and hyperplasia of the acinar cells may occlude the lumen giving rise to islands of plump cells in the lymphocytic stroma. Distended blood vessels and thickening of the connective tissue capsule around the thyroid are also seen.

Severe thyroiditis (+++).—Thyroids having lesions of this severity can be distinguished by their pale pink or white colour. In section the normal architecture is almost completely lost and the follicles are widely separated by the oedematous and fibrous lymphocytic stroma (Fig. 4). Very few follicles retain colloid and in most instances the lumen is infiltrated by mononuclear and, to a lesser extent, polymorphonuclear cells; the remainder are recognizable as cellular islands in the stroma which is essentially of a lymphocytic and histiocytic character with scattered polymorphonuclear leucocytes and plasma cells. Mast cells are always present in the normal gland but with increasing tissue damage they become degranulated and can rarely be distinguished in severe lesions. The epithelial

cells frequently show mitotic figures coupled with an increase in cell height throughout the gland. The capillary network is obliterated.

Periodic acid-Schiff (PAS) staining has been applied to sections of thyroiditis glands in an attempt to demonstrate the fate of thyroid colloid which reacts strongly with this reagent. Most of the polymorphonuclear cells show uniform cytoplasmic staining while histiocytes characterized by irregular-shaped nuclei frequently contain globules of PAS-positive material ; the more regularly shaped histiocytes are unstained. Intensely staining material cannot be detected in the interstitial spaces. Occasionally epithelial cells with extremely vacuolated cytoplasm appear to have been invaded by the inflammatory cells (Figs. 10 and 11). Associated with this phenomenon there may be invasive cells in the lumen of the follicle and evidence of colloidophagy (Fig. 12).

Severe thyroiditis with goitre (++++).—The glands are pale and visibly enlarged. Specimen glands with thyroiditis of this grade after fixation in formol saline and dehydration in absolute alcohol were dissected off the trachea and weighed up to 44 mg. Similarly treated thyroids from normal rats weighed 6–13 mg. Histologically, the changes are even more extreme than in a +++ thyroiditis : no intact follicles remain and the thyroid capsule becomes oedematous and infiltrated (Fig. 5). In some instances the gland may be difficult to dissect owing to its fusion with the muscle, trachea and oesophagus (Fig. 6). The inflammatory reaction extends through the capsule to the perithyroid muscle tissue where the lesion is often characterized by a predominance of polymorphonuclear leucocytes.

Effects of immunization with different thyroid antigens

The results obtained by injecting rats with homologous and heterologous thyroid antigens in Freund's adjuvant are presented in Table I which summarizes the frequency and variation in severity of the induced thyroiditis.

TABLE I.—*Severity of Thyroiditis Obtained on Injection of Various Substances in Freund-type Adjuvant*

	Substance injected*	Boosting injection at 3 weeks	Period between, first injection and examination	Number of animals	Severity of histological lesions†					Mean‡ score
					–	+	++	+++	++++	
Rat	thyroglobulin	–	13–16 days	25	5	7	3	1	9	2·1
Rat	thyroid extract	–	13–21 days	4	1	0	2	1	0	1·7
Rat	thyroglobulin	+	4 weeks	20	1	1	4	2	12	3·1
Rat	thyroid extract	+	,, ,,	42	4	2	10	6	20	2·9
Human	thyroglobulin	+	,, ,,	11	0	1	8	2	0	2·1
Sheep	thyroglobulin	–	13–21 days	45	16	11	15	2	1	1·1
Sheep	thyroglobulin	+	4 weeks	5	0	0	4	0	1	2·4
Sheep	thyroid extract	+	,, ,,	5	1	1	3	0	0	1·4
Saline		–	6–20 days	12	10	2	0	0	0	0·2
Saline		+	4 weeks	20	19	1	0	0	0	0·1
Rat	serum	+	4 weeks	5	3	2	0	0	0	0·4
Rat	kidney extract	+	4 weeks	5	5	0	0	0	0	0
Rat	adrenal extract	+	4 weeks	5	5	0	0	0	0	0
Rat	pituitary extract	+	4 weeks	5	5	0	0	0	0	0

* Injected dose contained 0·013 ml. of saline tissue extract (1 : 1 w/v) or 2·0 mg. thyroglobulin in adjuvant. Where indicated the animals were boosted with an identical injection.

† The lesions were graded as described in the text.

‡ Lesions were scored as follows : – = 0 ; + = 1 ; ++ = 2 ; +++ = 3 ; ++++ = 4 ; the arithmetic mean score is given.

While human and sheep thyroglobulin are capable of evoking thyroiditis, the homologous antigen is clearly more effective. Immunization with purified thyroglobulin preparations gave a higher thyroiditis score than thyroid extract. More intense lesions were found when the animals were given a second injection at 3 weeks as compared with those receiving only a single immunizing injection. Thus, of the different experimental treatments studied, immunization with homo-

EXPLANATION OF PLATES

FIG. 1.—Normal appearance of rat thyroid. ×96.

FIG. 2.—Thyroiditis (grade +) in thyroid of a rat immunized with 2 mg. sheep thyroglobulin with boosting 3 weeks later and examination at 1 month. Two foci of inflammation, the larger one involving the breakdown of 3 acini, and some oedema are visible. (×96.)

FIG. 3.—Thyroiditis (grade ++) in thyroid of a rat immunized with a single injection of 2 mg. sheep thyroglobulin and examined 19 days later. (×140.)

FIG. 4.—Thyroiditis (grade +++) in thyroid of a rat immunized with rat thyroid extract with boosting 3 weeks later and examination at 1 month. The gland is diffusely and heavily infiltrated throughout. (×96.)

FIG. 5.—Thyroiditis (grade ++++ with goitre) in thyroid of a rat immunized with 2 mg. rat thyroglobulin, boosted at 3 weeks and examined at 1 month. There is heavy infiltration with complete absence of a normal follicular structure. Cellular nests in the lymphocytic stroma represent the follicular remnants. The oedematous capsule seen at the bottom of the picture is also invaded. (×96.)

FIG. 6.—Severe thyroiditis in a rat immunized with 2 mg. rat thyroglobulin, boosted at 3 weeks and examined at 1 month. The thyroid lobes consist of dense cellular masses of disorganized follicular cells in a heavy lymphocytic stroma. One lobe is greatly enlarged. The muscles, trachea and oesophagus are fused with the thyroid by an oedematous, fibrous matrix heavily infiltrated by polymorphonuclear leucocytes. (×14.)

FIG. 7.—Thyroiditis in a rat immunized with 2 mg. sheep thyroglobulin, boosted at 3 weeks and examined at 1 month, showing perivascular infiltration, predominantly lymphocytic, associated with follicular changes. (×350.)

FIG. 8.—Early lymphocytic focus in a rat thyroid 13 days after immunization with 2 mg. sheep thyroglobulin showing scattered lymphocytes, histiocytes and polymorphonuclear cells with follicular invasion. (×359.)

FIG. 9.—Cellular infiltrate in the thyroid of a rat immunized with 2 mg. rat thyroglobulin with boosting. The stroma is moderately fibrous and populated by histiocytes, lymphocytes and a few polymorphs. There is complete degeneration of the follicles. A blood vessel is seen packed with mononuclear and polymorphonuclear cells. (×718.)

FIG. 10–12.—Thyroid of a rat obtained 15 days after immunization with 2 mg. sheep thyroglobulin showing stages in the invasion of the epithelial cells and the follicular lumen. The basement membrane of the follicle is indicated by arrows :

FIG. 10.—The heavily infiltrated stroma surrounds the follicles. A few epithelial cells are shown which are greatly swollen and vacuolated, and appear to contain nuclei of inflammatory cells in addition to the nuclei of the epithelial cells. (×430.)

FIG. 11.—All cells of the thyroid epithelium are swollen, vacuolated and invaded by cells of the inflammatory exudate. The colloid shows peripheral vacuolation but does not contain invading cells. (×345.)

FIG. 12.—In addition to the invaded appearance of the follicular epithelial layer the lumen is intensively infiltrated and the colloid is almost entirely lost. (×345.)

FIG. 13.—Thyroid tissue from a rat immunized with rat thyroid extract and boosted 21 days later illustrating regression of thyroiditis.

(*a*) Appearance of one thyroid lobe removed by hemithyroidectomy 7 days after boosting. There is heavy infiltration between the follicles and some invasion of follicles by histiocytes and lymphocytes. (×265.)

(*b*) The remaining lobe of the thyroid examined after a further 19 days is normal. (×265.)

FIG. 14.—Thyroid tissue from a rat immunized with rat thyroglobulin and boosted 21 days later illustrating regression of thyroiditis.

(*a*) Appearance of one thyroid lobe, which was much enlarged, removed by hemithyroidectomy 7 days after boosting which shows severe inflammation and almost complete loss of the follicular pattern. (×265.)

(*b*) The remaining lobe of the thyroid examined after a further 50 days showing regeneration of the follicular pattern with marked loss of inflammation. (×265.)

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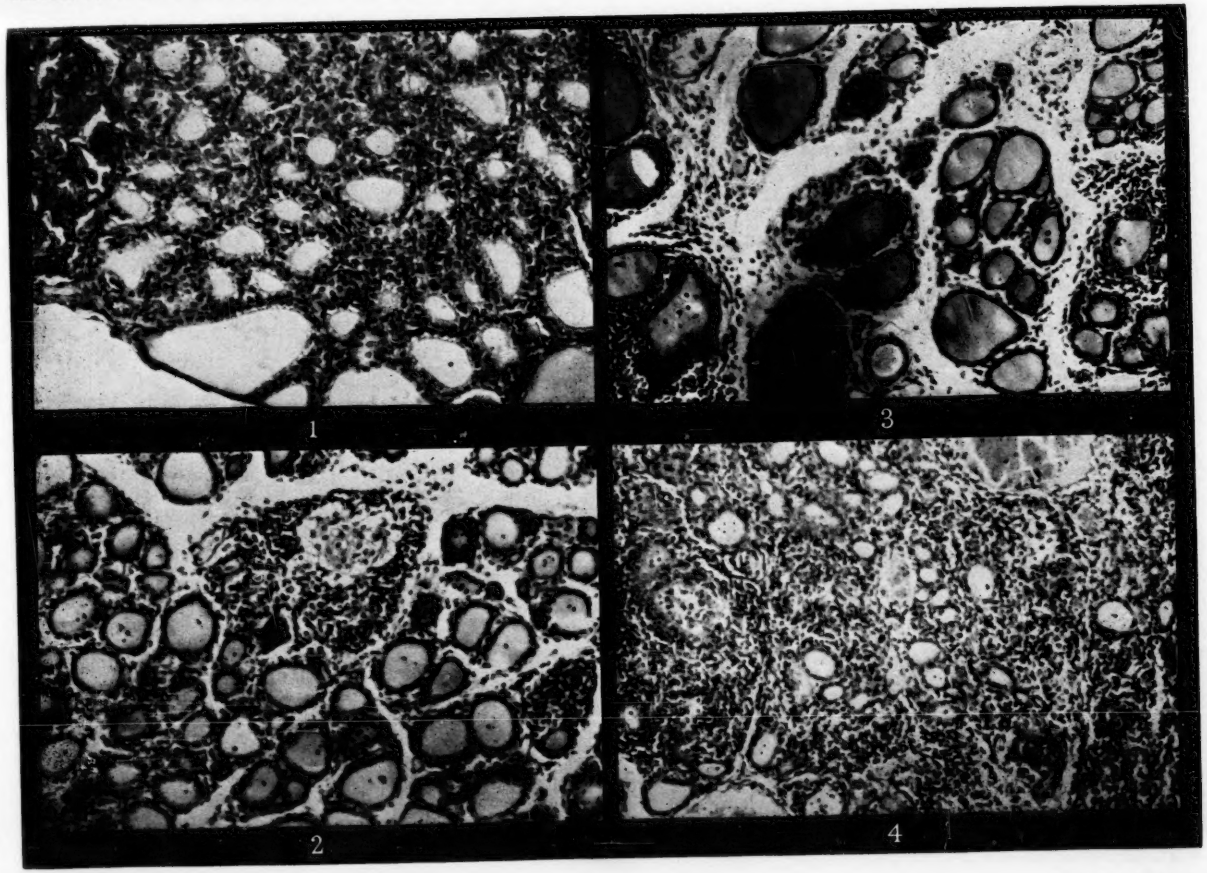
BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.



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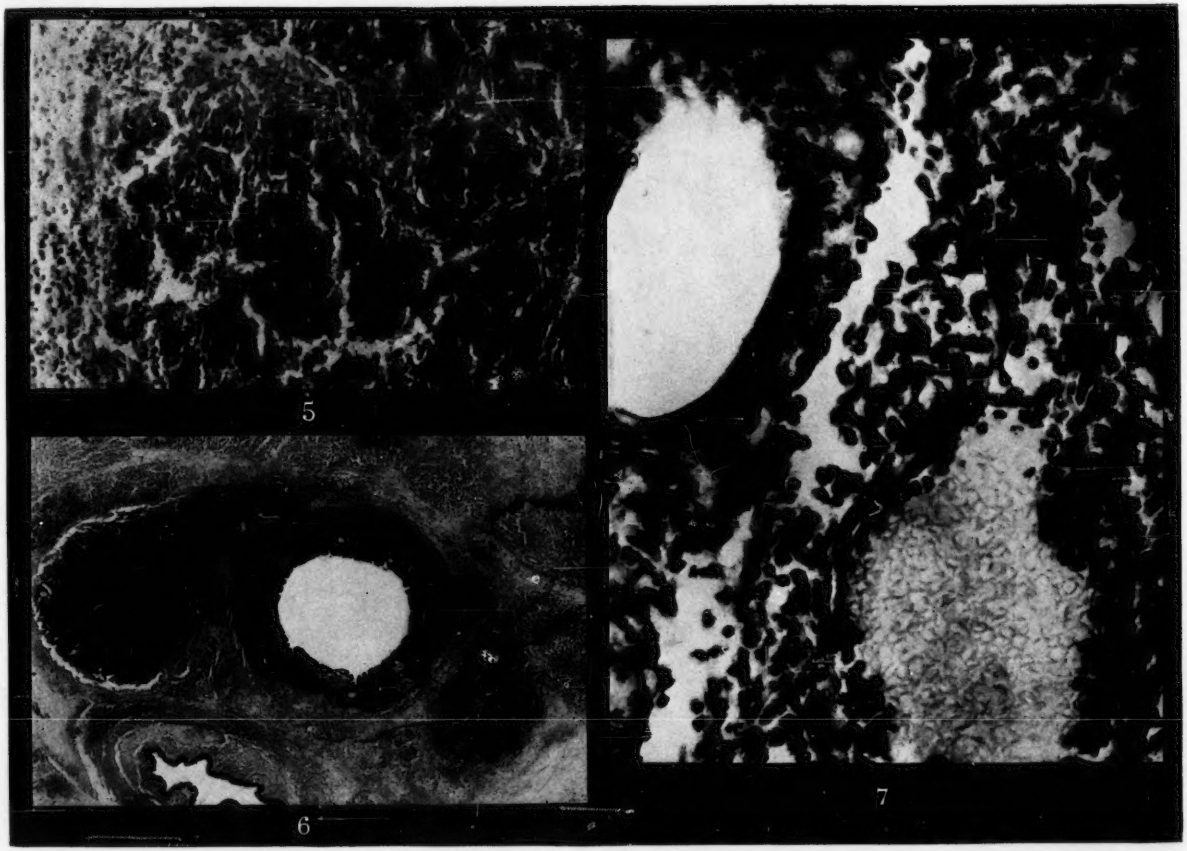

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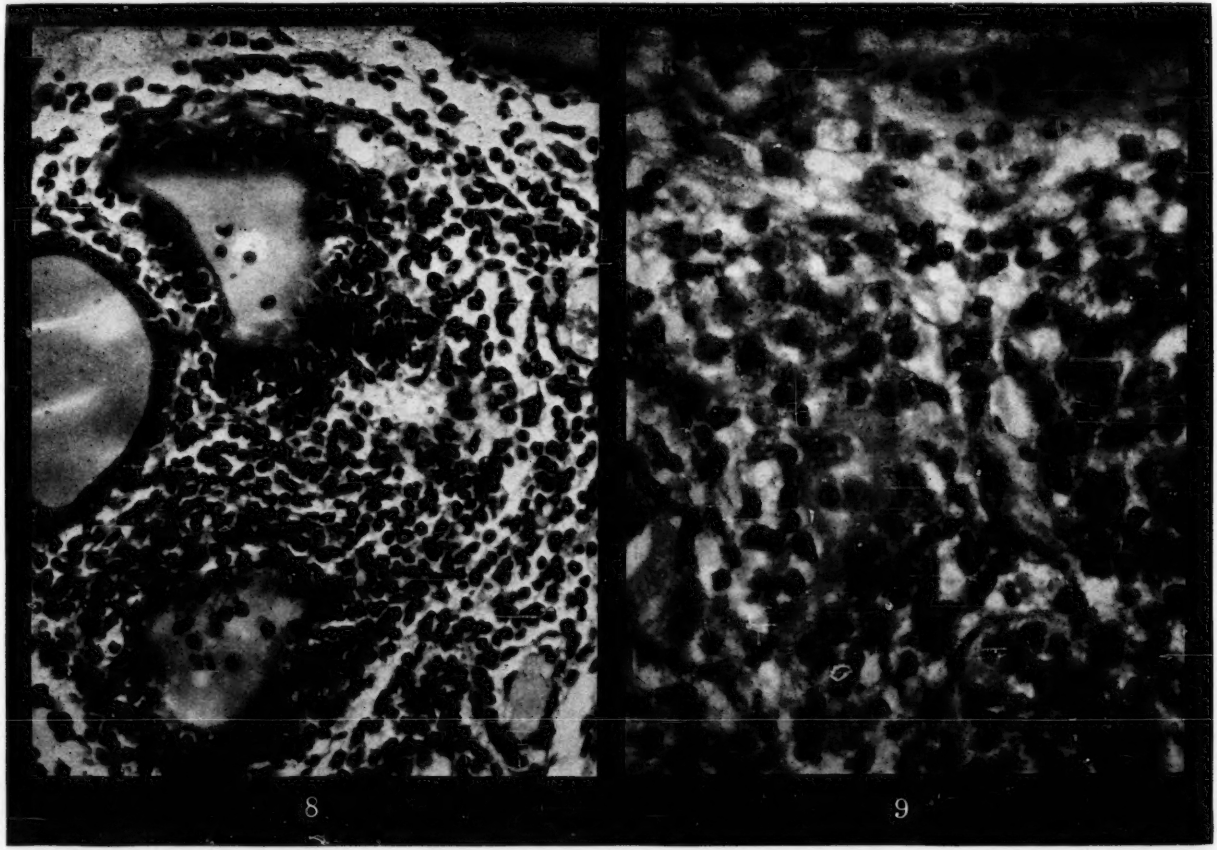

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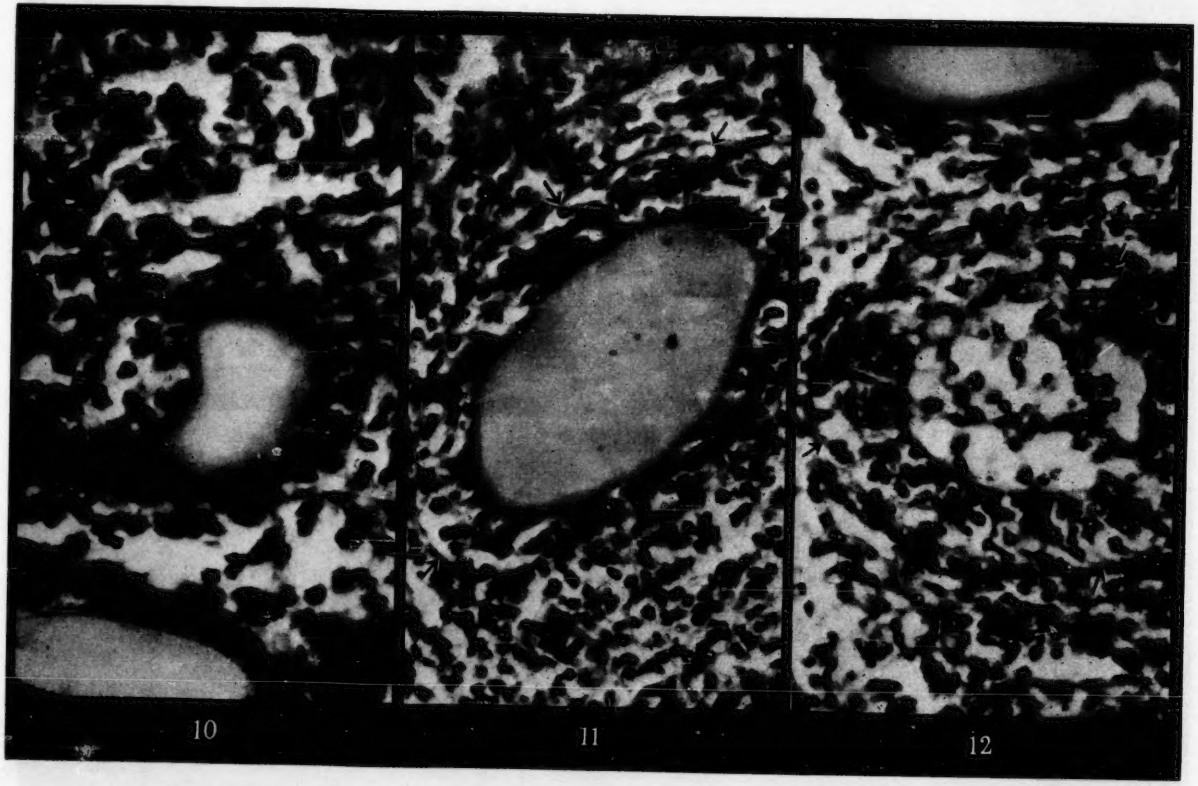

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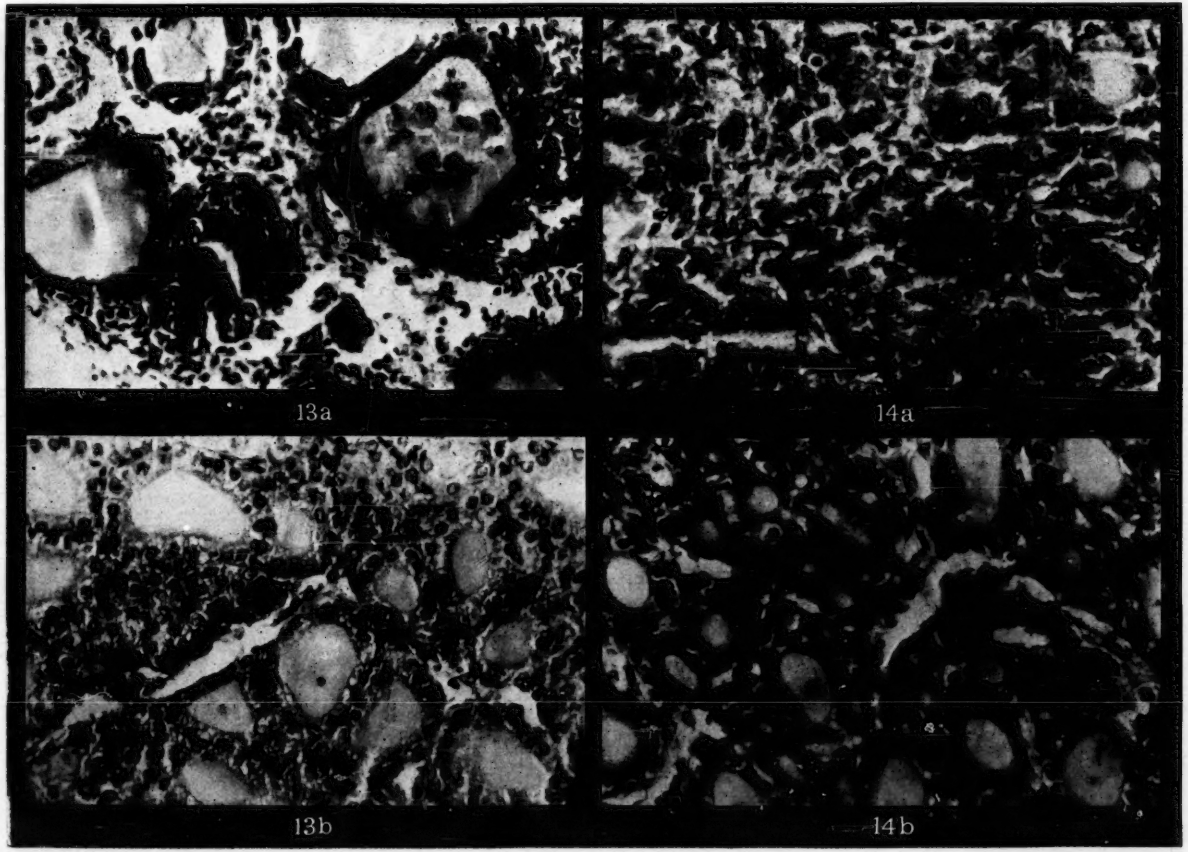

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logous antigen, in particular with rat thyroglobulin, followed by a boosting injection was the most effective for the consistent production of severe thyroiditis.

Groups of control rats were injected with saline, rat serum or extracts of rat kidney, adrenal or pituitary, all in Freund's adjuvant. In a very small percentage of animals, minimal thyroiditis lesions were produced but diffuse changes were never encountered. "Rheumatoid" swellings frequently affected the limbs of rats injected with Freund adjuvant in both control and thyroid immunized groups (Pearson and Wood, 1959). There were no obvious differences between the modified and the "classical" adjuvant mixtures in their effectiveness for the production of thyroiditis. No significant thyroid lesions were seen after immunization with the auto-antigen in incomplete Freund's adjuvant lacking the acid-fast bacilli.

Effects of variation in the amount of thyroglobulin injected are summarized in Table II. Examination of the thyroids 15 days after a single injection showed

TABLE II.—*Severity of Thyroiditis Obtained 15 days After Injection of Different Amounts of Rat Thyroglobulin in Adjuvant*

Amount injected	Number of animals	Severity of histological lesions					Mean score
		−	+	++	+++	++++	
2·0 mg.	25	5	7	3	1	9	2·1
200 μg.	11	4	4	1	1	1	1·2
20 μg.	5	3	2	0	0	0	0·4
2 μg.	5	4	1	0	0	0	0·2

Results are expressed as in Table I.

that 200 μg. of rat thyroglobulin could produce clear-cut thyroiditis; a 2·0 mg. dose was more effective in that lesions were found in a higher proportion of the animals and were of greater severity. Levels of 20 μg. or less were marginally effective although on boosting significant thyroiditis resulted.

Immunization with homologous thyroid antigens led to the appearance of circulating antibodies detectable by their reaction with thyroglobulin-coated tanned red cells; these sera did not deviate complement in the presence of thyroglobulin. After boosting, precipitins were demonstrated in a number of sera using Oudin's gel diffusion technique. Skin reactions could not be elicited by intradermal challenge with thyroid extract 2 weeks after immunization although delayed hypersensitivity to PPD was demonstrable in many adjuvant treated animals and was characterized by induration without erythema following intradermal injection of antigen.

Development of thyroiditis with time

Reasonably severe thyroiditis was found as early as 10 days after immunization with rat thyroglobulin and was not more pronounced by the 15th day (Table III). Following a single injection of 2 mg. of sheep thyroglobulin in adjuvant, lesions appeared at the 10th day, reached a maximum at 16 days and then began to decline in severity. This suggested that the thyroiditis might be reversible and further experiments were carried out to test this possibility.

Rats were immunized with 2 mg. rat thyroglobulin and given a booster injection at 21 days. The animals were hemi-thyroidectomized on the 28th day and

TABLE III.—*Development of Thyroid Lesions at Different Times After a Single Injection of 2·0 mg. of Thyroglobulin in Adjuvant*

Antigen	Days after injection	Number of rats	Severity of histological lesions					Mean score
			−	+	++	+++	++++	
Rat thyroglobulin	10	5	1	1	1	0	2	2·2
	13	5	0	2	1	1	1	2·2
	15	25	5	7	3	1	9	2·1
Sheep thyroglobulin	7	2	2	0	0	0	0	0
	10	7	2	5	0	0	0	0·7
	13	18	9	5	4	0	0	0·7
	15	15	6	3	4	1	1	1·2
	16	3	0	1	2	0	0	1·6
	21	6	1	2	3	0	0	1·3
	29	4	2	2	0	0	0	0·5

Results are expressed as in Table I.

killed after various time intervals. The histology of the thyroid lobe excised at the 28th day was compared with that found in the remaining lobe at death (Table IV). Regression of the lesions with time was a consistent feature throughout

TABLE IV.—*Regression of Auto-immune Thyroiditis with Time*

Days between hemithyroidectomy on 28th day after immunization and death	Rat number	Inflammatory cell reaction		Follicular damage	
		On 28th day	At death	On 28th day	At death
4	159	+++	+++	+++	+++
6	142	+	+	+	+
	145	±	−	±	−
12	160	+++	+	++	+
	144	+	−	−	−
	164	+++	+	+++	+
19	154	++	−	+	−
	162	++	−	++	−
	165	+++	+	++	++
20	147	+	−	+	−
30	155	+	−	+	−
	156	++	+	+	−
	157	+	−	++	−
	158	+	−	++	−
	166	++	+	++	−
33	146	+++	−	++	+
41	167	+++	−	++	−
50	163	++++	+	++	±
	168	++++	+	+++	−

Rats were immunized with 2 mg. homologous thyroglobulin in adjuvant B and boosted at 21 days.

the group of animals studied (Figs. 13*a* and *b*). Significant regression of both the inflammatory cell reaction and the degree of follicular damage was observed as early as 12 days after hemi-thyroidectomy (*i.e.* 19 days after the boosting injection). One animal showing severe thyroiditis at hemi-thyroidectomy had a normal gland when examined 41 days later. In a further 2 rats, severe thyroiditis

with gland enlargement resolved to give a thyroid with normal architecture and occasional foci of inflammatory cells by the 50th day after hemi-thyroidectomy (Figs. 14*a* and *b*). After recovery from thyroiditis, the glands tended to be larger than normal and showed numbers of small round colloid-containing follicles with hyperplastic epithelium. An increase in the fibrous interfollicular tissue was the only indication of a previous lesion.

DISCUSSION

Following the methods used by Rose and Witebsky (1956) for the production of auto-allergic thyroiditis in rabbits, the first studies with rats were made with repeated intradermal injections of rat thyroid extract emulsified in the standard complete Freund's adjuvant. However on histological examination of the thyroids 3–5 months after the initial treatment no inflammatory lesions could be seen and attempts were made to find conditions which would lead to thyroiditis in this species. In subsequent experiments the composition of the adjuvant was altered empirically and the time interval between treatment and examination shortened. A single injection of homologous thyroid extract emulsified in the modified adjuvant followed by a booster injection at 3 weeks, led to the production of thyroiditis in 90 per cent of the animals examined 1 week later. Under these conditions the standard adjuvant and the modified preparation gave comparable results. It was further shown that thyroid lesions were present in up to 80 per cent of the animals 10–15 days after a single immunizing dose of 2 mg. of a rat thyroglobulin preparation. While thyroiditis which was classed as definite or severe could also be provoked by 200 μg. of thyroglobulin, smaller amounts gave only mild lesions. The 1 : 1 (w/v) saline extract of rat thyroid was less effective than 2 mg. of purified thyroglobulin, largely as a result of its lower thyroglobulin content but also perhaps because of competition between the thyroglobulin and other tissue constituents present.

Both sheep and human thyroglobulin can evoke significant thyroiditis in the rat suggesting that these proteins possess configurational groupings structurally similar to the autoantigenic determinants on the homologous antigen. Although the heterologous proteins were not as effective as rat thyroglobulin, the disparity in auto-antigenic potency was less marked when the boosting schedule was used as evidenced by the mean thyroiditis scores of 3·1, 2·4 and 2·0 for rat, sheep and human thyroglobulin respectively (see Table I). Witebsky and Rose (1959) have reported the occurrence of thyroiditis in rabbits after immunization with hog thyroglobulin but with this species combination, relatively intensive treatment was required.

The ability of Hashimoto sera to precipitate 96 per cent of the radioactivity of human [^{131}I]thyroglobulin (Roitt and Doniach, 1959) demonstrates that thyroglobulin is an auto-antigen and therefore should be capable of evoking the auto-allergic disease. However, auto-antibodies to a second colloid antigen (CA2) distinct from thyroglobulin have been described recently (Balfour, Doniach, Roitt and Couchman, 1961 ; Roitt, Doniach, Wilson and Couchman, 1960) and are sometimes found in Hashimoto sera when tests for thyroglobulin and microsomal antibodies are negative. It is pertinent to note that the majority of thyroglobulin preparations studied contain bound CA2 and one cannot yet exclude the possibility that this antigen could be primarily responsible for the chain of events leading to

destruction of the thyroid. Further purification of the active antigen(s) must be undertaken to settle this point. The relative inefficiency of whole thyroid extracts to evoke thyroiditis in the rat would indicate that the antigen is derived from the soluble rather than the particulate fraction and preliminary attempts to induce thyroiditis with a " microsomal " preparation have so far been unsuccessful.

It is well known that the Freund's adjuvant itself is capable of producing widespread pathological phenomena (Pearson, Waksman and Sharp, 1961). In the present studies the control animals injected with adjuvant alone, or in combination with rat serum or saline extracts of rat adrenal, kidney or pituitary showed a variety of changes which in the thyroid usually took the form of a generalized vascular dilatation. Occasionally, perivascular cuffing with mononuclear cells was observed but this occurred mostly in the perithyroidal connective tissue and muscle. In 5 out of the 52 control animals, focal inflammatory lesions associated with follicular breakdown were seen. Such effects may represent the extreme end of a spectrum of generalized tissue changes induced by the adjuvant which cannot readily be differentiated from the minimal (grade +) lesions obtained as a specific response to thyroid antigens. However, the more diffuse (grade ++) thyroiditis was never encountered in the control animals and it is clear that inclusion of the thyroid antigen in the adjuvant greatly increases the severity of the lesions (Table I) and in practice the specific effects of auto-immunization are easily recognized. The role of Freund's adjuvant in the pathogenesis of the experimental auto-allergies has not been clearly defined and it has been the subject of considerable speculation including the suggestion that it may impair the homeostatic mechanisms controlling immune tolerance. In addition to its ability to enhance antibody synthesis and to potentiate delayed hypersensitivity it is conceivable that the adjuvant may also act by producing permeability changes which could make the tissue antigens more accessible to immunologically competent cells or circulating antibody.

It is of considerable interest that in certain glands showing thyroiditis there was an apparent invasion of epithelial cells by lymphocytes and histiocytes with vacuolation of the cytoplasm and one may speculate that this represents a mode of attack by sensitized inflammatory cells upon their target rather than penetration of already damaged cells. Similar phenomena have been reported by Darcy (1952) in the rejection of thyroid homografts in the rabbit, and by Waksman (1960*a*) in delayed dermal hypersensitivity reactions. The penetration of epithelial cells by healthy lymphocytes has been observed in cultures of Hashimoto goitres (Pulvertaft, Doniach, Roitt and Hudson, 1959), in rat sarcoma cultures (Koller and Waymouth, 1953) and in other contexts (Trowell, 1958).

That white cells may be instrumental in bringing about the tissue breakdown seen in auto-immune thyroiditis is suggested by certain histological features of the affected glands. Thus our finding of follicular degeneration in the presence but not in the absence of invading inflammatory cells would accord with this view although it might be argued that in animals showing delayed hypersensitivity, the occurrence of tissue destruction independently of lymphoid cells might be difficult to detect if these cells are able to migrate very rapidly to affected sites. Again, in mild early lesions, the inflammatory exudate is predominantly lymphocytic and histiocytic with some plasma cells appearing in the more diffuse (grade ++) thyroiditis. Significantly, polymorphonuclear cells are rare or absent in these lesions the histological features of which are thus characteristic of a delayed

hypersensitivity response (Gell and Hinde, 1951), known to be mediated by white cells. Unfortunately, dermal reactions to thyroid antigens could not be evoked 2 weeks after the initial immunization although delayed hypersensitivity to PPD could be demonstrated. Delayed dermal reactions are readily elicited in guinea-pigs and rabbits similarly immunized with homologous thyroglobulin in Freund's adjuvant, and Waksman (1960*b*) has suggested that the difficulties encountered in attempting to demonstrate tuberculin type sensitivity in the rat may be ascribed to the almost complete absence of a venular network in the dermis of the flank.

The ability of cells rather than serum to evoke auto-allergic lesions in a normal recipient on transfer from an actively immunized animal has been demonstrated by Paterson (1960) in experimental allergic encephalomyelitis. Lymph node cells from rats injected with an encephalitogenic antigen in Freund's adjuvant were passed to recipient animals tolerant of a donor graft, and gave rise to limited histological lesions and to paralysis in a large proportion of the transfers. Further evidence for an aggressive role of " sensitized " leucocytes against target cells may be cited from tissue transplantation studies in which it has been possible to demonstrate the development of delayed hypersensitivity reactions to donor transplantation antigens in guinea-pigs homografted with skin (Brent, Brown and Medawar, 1958) and the rejection of homografts originally surviving in tolerant animals, by " adoptive immunity " using isogenic lymphoid cells (Billingham, Brent and Medawar, 1954). Using grafts enclosed in diffusion chambers with different pore sizes, Algire, Weaver and Prehn (1954) showed that the tissue was destroyed in chambers which allowed access of white cells and survived in those which excluded leucocytes. Govaerts (1960) has described experiments in which thoracic duct cells and serum from a dog which had rejected a kidney homograft were added to tissue cultures of the second kidney of the donor and produced retraction, agglutination and granulation of the cytoplasm and in some instances death of the epithelial cells. There is a strong case for an active role of lymphoid cells in the pathogenesis of autoimmune lesions and in graft rejection, and perhaps an equally strong one against a dominant role for serum antibody, but one cannot preclude the possibility that both the cellular and the humoral elements of the immune response operate synergistically. The studies quoted do not resolve this question since even in transfer experiments the recipient acquires cells with the ability to produce circulating antibody as well as to confer delayed-type sensitization.

Hyperplasia of the thyroid epithelial cells is commonly seen during the development of auto-immune thyroiditis in the rat and is most intense in the severest lesions. Localized hyperplasia frequently occurs in relation to focal areas of lymphoid infiltration presumably as a consequence of the inflammatory process and it may be that these changes precede tissue breakdown. In other instances generalized hyperplasia with colloid vacuolation has been observed in glands showing minimal or no thyroiditis suggesting stimulation by an underlying humoral mechanism.

Severely affected thyroids were greatly enlarged and in these instances, particularly in boosted animals having high circulating antibody titres, extra-thyroidal inflammation may occur, sometimes leading to fusion of the gland with the surrounding structures. Polymorphonuclear leucocytes were abundant in these lesions and may possibly be indicative of an Arthus-like reaction between circulating anti-

body and colloid leaking from the extensively damaged gland. This is of interest in view of the extrathyroidal invasion seen in cases of human thyroiditis, particularly in Riedel's struma.

The intensity of thyroiditis following a single injection of sheep thyroglobulin reached a maximum at 16 days and became less severe by 4 weeks (Table III) suggesting that the original failure to observe thyroiditis 3–5 months after auto-immunization was due to regression of the lesions. This was confirmed by experiments in which thyroiditis was proved by hemithyroidectomy 1 week after a booster injection and the remaining lobe of the gland examined 1–7 weeks later. Minimal lesions disappeared in about 12 days but extensive lesions required several weeks. The architecture of the regenerated gland was normal with some residual fibrosis. Recovery has also been reported in allergic encephalomyelitis in animals and in human post-rabies vaccination encephalitis. Evidently the immunization procedure has failed to induce the progressive destruction which characterizes extreme cases of Hashimoto's disease and primary myxoedema, and in general the experimental auto-allergies do not parallel the sustained nature of the human diseases which they model. It was earlier postulated that in Hashimoto's disease a vicious spiral of tissue damage and consequent boosting of the auto-immune response by liberated antigen was responsible for progression of the lesions (Doniach and Roitt, 1957) but this hypothesis requires some modification in view of the finding that lymphadenoid change in the human is not always progressive (Vickery and Hamlin, 1961) and the present demonstration of the transient nature of experimental auto-immune thyroiditis in the rat. The factors which distinguish the progressive from the stationary or regressive lesions provide a challenge for further enquiry.

SUMMARY

Marked thyroiditis may be evoked consistently in the rat by immunization with homologous or heterologous thyroid extract or thyroglobulin in Freund adjuvant. Lesions are seen at 10 days. In severe lesions the entire thyroid becomes diffusely infiltrated by inflammatory cells, chiefly histiocytes and lymphocytes, and there is widespread breakdown of the acinar architecture with rupture of the follicular epithelium. The glands increase in weight up to 5 times the normal. In some instances the lesions extend into the perithyroidal tissues. The character of the thyroid lesions is consistent with the view that tissue destruction is mediated primarily by the invading inflammatory cells although one cannot exclude the possibility that serum antibody is implicated, perhaps in a synergistic role. Regression of thyroiditis occurs regularly and this contrasts with the chronic, usually unremitting course of the human disease which it models.

We thank Professor Sir Charles Dodds and Professor F. Dickens for their unfailing support. We are particularly grateful to Dr. D. Doniach and Dr. D. Senhauser for helpful discussions. Dr. J. Colover kindly provided a sample of his bacillary residues. Our thanks are due to Mr. E. D. Decker, Miss P. Wilson and Mr. C. Shapland for their invaluable technical assistance and to Mr. P. A. Runnicles for taking the photographs. The work has been supported by grants to the Medical School from the British Empire Cancer Campaign.

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AN EFFECT OF THE GRAFT-VERSUS-HOST REACTION ON RESISTANCE TO EXPERIMENTAL BACTERAEMIA

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Received for publication May 19, 1961

A GRAFT-VERSUS-HOST reaction develops when immunologically competent cells are introduced into an antigenically foreign host which, for any reason, is unable to reject them. (See review by Simonsen, 1961.) In man, for example, this situation can arise when homologous bone marrow or spleen cells are transplanted into an individual treated with ionizing radiation or radiomimetic or antimetabolite drugs at a dosage level sufficient to depress the homograft reaction. For studying the effects of graft-versus-host reaction experimentally, however, it is preferable where possible to avoid such potentially complicating factors as irradiation. In the experimental system used in the work described here, the recipient is *genetically* tolerant of the donor cells. When a first-generation (F1) hybrid between two inbred strains of mice is injected with splenic or lymphoid cells from either of the parent strains, graft-versus-host reaction develops unopposed, for all the antigens of the donor are present in the recipient. The donor cells attack the host because it contains antigens inherited from the other parent strain.

Several changes demonstrated recently in animals undergoing graft-versus-host reaction of this type suggested that there might be some accompanying modification in resistance to bacterial infection. Two weeks after the injection of parental strain spleen cells into F1 hybrid mice, the phagocytic activity of the reticulo-endothelial system (RES) is intensely stimulated, as assessed by the rate of clearance of colloidal carbon from the blood (Howard, 1961*a*). At the time of this RE response, the susceptibility of mice to the lethal effect of bacterial endotoxin is enormously increased (Howard, 1961*b*)—an effect analogous to certain other instances in which the RES has been stimulated. Furthermore, in mice with a severe graft-versus-host reaction, formation of humoral antibody to *Salmonella typhi* H antigen has been found to be depressed (Howard and Woodruff, 1961).

We have now studied the effect of graft-versus-host reaction during its early proliferative phase on the resistance of mice to two bacterial pathogens—*Diplococcus pneumoniae* and *Salmonella typhimurium*. Although both are virulent for the mouse, their respective fates *in vivo* show characteristic differences. *Salm. typhimurium* is readily phagocytosed but survives and multiplies within RE cells. On the other hand, although *D. pneumoniae* is rapidly killed within RE cells, it proliferates extracellularly, being normally very resistant to phago-

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cytosis in the absence of antibody. Stimulation of the RES by bacterial endotoxin, however, has been found to promote rapid phagocytic clearance of *D. pneumoniae* from the blood, when the virulence of the organism had been moderately reduced by brief exposure to 0·1 M phosphate buffer *in vitro* (Cooper and Stuart, 1961 ; Cooper and Stuart, 1962). The same model of bacteraemic infection has been repeated here and an analogous effect obtained at a time when the RES was stimulated by a graft-versus-host reaction instead of endotoxin.

MATERIALS AND METHODS

Mice.—The animals used were adults (20–30 g.) belonging to the strains A, C57BL and CBA, and F1 hybrids derived from them. In any one experiment, the body weights did not vary by more than 5 g. Female spleen donors were used throughout, but in some experiments the recipients were male and in others female. (Stimulation of the phagocytic activity of the RES occurs equally well in all four donor-recipient sex combinations.)

Preparations of spleen cell suspensions.—Spleens were broken up gently in pH 7·4 Hanks' solution by means of a loose-fitting ground glass homogenizer. The coarse suspension was filtered through a fine-meshed stainless steel sieve and centrifuged at 150 *g* for 7 min. The supernatant was discarded and the cells were resuspended in fresh Hanks' solution by repeatedly sucking in and out of a Pasteur pipette. Large particles were allowed to sediment out before the supension was injected into a tail vein. Cell counts were made in a haemocytometer using white cell diluting fluid.

Quantitative estimation of the phagocytic activity of the reticulo-endothelial system (RES).—The rate of disappearance from the blood of a standard dose of stable colloidal carbon (16 mg./100 g. body weight, preparation C11/1431a) was measured as described by Biozzi, Benacerraf, Stiffel and Halpern (1954). After the carbon was injected into a tail vein, repeated blood samples (0·05 ml.) were removed from the retro-orbital venous plexus, lysed in 0·1 per cent Na_2CO_3 and the amount of carbon estimated in a photoelectric absorptiometer. The blood clearance of carbon follows an exponential function, so that the phagocytic index " K " =

$$\frac{\log \text{concentration } a - \log \text{concentration } b}{tb - ta}$$

where ta and tb = time in min. at a and b.

Pneumococcal infection.—A strain of *Diplococcus pneumoniae* type 8 " L " was used. It was recovered from the dry state by culture in horse digest broth for 18 hr. at 37°. " Stock " suspensions of organisms for preparing challenge inocula were made by 100-fold dilution of the culture in horse digest broth. Immediately before injection of each group of 10 mice, two further serial 10-fold dilutions of the " stock " suspension were made in 0·1 M phosphate buffer (pH 7·2). 0·1 ml. volumes of the final dilution were injected intravenously. Bacterial counts on the inocula used for each group were made by the technique of Miles and Misra (1938). Fuller details of the pneumococcal infection are being published elsewhere (Cooper and Stuart, 1962).

Determination of the concentration of pneumococci in the blood, liver and spleen.—Groups of 5 animals were used. The animals were infected with approximately 8×10^3 organisms suspended in phosphate buffer. Immediately after injection, then at 30 min., 1, 2, 5, 8, 24, 32 and 48 hr. afterwards samples of blood were withdrawn from the retro-orbital venous plexus. The mice were killed at the 48th hr. of infection, and the livers and spleens removed with aseptic precautions and washed in phosphate buffer. Individual homogenates of the organs were made in 5 ml. volumes of phosphate buffer, using ground-glass tissue grinders. The numbers of viable organisms in the blood and tissue homogenates were determined by the Miles and Misra method.

Salmonella typhimurium *infection.*—A virulent, non-fimbriate strain of *Salmonella typhimurium* (S 6683) was supplied by Dr. J. P. Duguid. The LD_{50} of this strain by the intraperitoneal route was approximately 20 organisms. The organism was maintained in the dry state and recovered in horse digest broth culture immediately before use. An 18-hr. culture, incubated at 37°, was serially diluted by two 10-fold steps in 0·1 M phosphate buffer.

The challenge dose for each mouse was 0·1 ml. of the last dilution. Viable counts were again used to determine the dose of organisms.

The bacterial origin of all deaths in both *Salm. typhimurium* and *D. pneumoniae* experiments was confirmed by heart blood cultures.

RESULTS

Graft-versus-host reaction was induced in two different hybrid host types (C57BL × A and C57BL × CBA) by intravenous injection of parental strain spleen cells (from A and C57BL strain donors respectively). In both cases the phagocytic activity of the RES was found to be increased, on average 8 to 10-fold, when determined 14 days later (Table I).

TABLE I.—*Stimulation of the Phagocytic Activity of the RES in F1 Hybrid Mice Injected with Parental Strain Spleen Cells 14 Days Previously*

F1 hybrid recipients	Spleen cells injected i.v. 14 days previously	Number of animals	Phagocytic index "K"
C57BL × A	63×10^6 A	18	0·180* (0·055–0·417)
	Nil	10	0·018 (0·014–0·022)
C57BL × CBA	66×10^6 C57BL	17	0·119 (0·087–0·192)
	Nil	20	0·014 (0·010–0·020)

* Mean value. Figures in brackets represent range of values found.

The resistance of mice to *D. pneumoniae* infection during this stage of graft-versus-host reaction was investigated in the same two hybrid types. The results given in Table II show that whereas intravenous injection of 1500 organisms into

TABLE II.—*The Effect of Graft-versus-host Reaction on the Resistance of Adult F1 Hybrid Mice to Infection with* D. pneumoniae *Type* 8 (1500 *Organisms I.V.*)

F1 hybrid	Dose of spleen cells 12 days previously (i.v.)	Number of survivors / Number injected	Mean time to death (days)
C57BL × A	70×10^6 A	10/10	—
	Nil	2/10	4·5
C57BL × CBA	70×10^6 C57BL	10/10	—
	Nil	2/10	3·5

untreated control mice of either type produced an 80 per cent mortality rate, similar mice given parental strain spleen cells 12 days before bacterial challenge were uniformly resistant. It seemed likely that this protective effect could be determined by more effective clearance of *D. pneumoniae* from the circulation by cells of the RES. The fate of *D. pneumoniae* was followed, therefore, for 48 hr. following intravenous injection of a suspension (9500 organisms) into two groups of 5 (C57BL × A) F1 mice, one control group and one treated with A strain spleen cells 12 days previously. A striking difference between the two groups

was found in their respective changes in blood concentration of bacteria (Fig.1). The control group showed a slow clearance of bacteria for the first 5 hr. only, followed by progressive increase in bacterial concentration up to a level approximately 100 times greater than that present initially. The spleen cell-treated group, on the other hand, were almost free from circulating bacteria within 8 hours and did not develop any secondary bacteraemia. This difference between the two groups was further emphasized by the virtually complete destruction of viable bacteria within the liver and spleen of those mice undergoing graft-versus-host reaction (Table III).

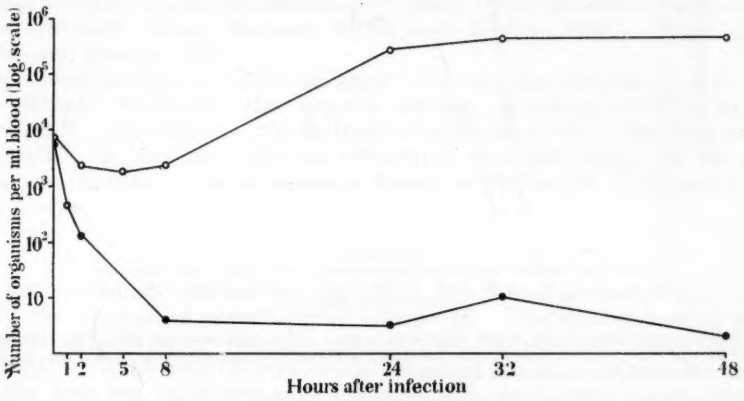

FIG. 1.—The effect of graft-versus-host reaction on the blood concentration of *D. pneumoniae* following the i.v. injection of 9500 organisms into (C57BL × A) F1 hybrid mice. Each curve represents the geometric mean readings obtained from a group of 5 animals.

○——○ Untreated controls.
●——● 70 × 10^6 A spleen cells 12 days previously.

TABLE III.—*The Effect of Graft-versus-host Reaction on the Number of Viable Organisms in the Liver and Spleen of* (C57BL × A) *F1 Mice* 48 *hours after I.V. Injection of* 9500 D. pneumoniae *Type* 8

Group	Number of viable organisms (geometric mean)	
	Liver	Spleen
Controls	2,100,000	650,000
70 × 10^6 A spleen cells 12 days previously	0	4

A dose of 250,000 *Salm. typhimurium* was injected intravenously into two groups of 15 (C57BL × A) mice, one group having received 70 × 10^6 A strain spleen cells 12 days previously. The pre-treated group showed no significant improvement in survival rate although the survival time of these mice was slightly increased as compared with the controls (Fig. 2). The 4 mice surviving at 21 days were not ill, but *Salm. typhimurium* was isolated from the liver and spleen of them all. Comparing harmonic mean death times (a measure which includes

information from the survivors), we have controls 7·0 days and treated mice 10·4 days. The difference is significant at the 5 per cent probability level ($t_{(28)} = 2{\cdot}29$).

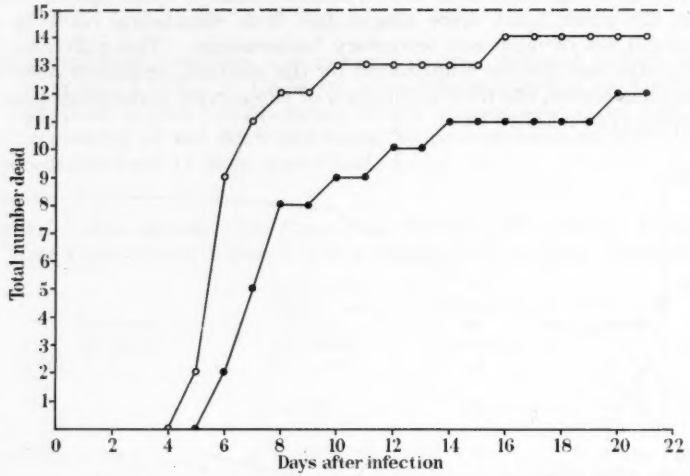

FIG. 2.—A comparison of the result of infection with *Salm. typhimurium* (250,000 organisms i.v.) in (C57BL × A) F1 hybrid mice with and without previous injection of A strain spleen cells. Each group contained 15 animals.

○——○ Untreated controls.
●——● 70×10^6 A spleen cells 12 days previously.

DISCUSSION

It should be emphasized that the above evidence of increased resistance to bacteraemic infection has been obtained during a relatively early stage of graft-versus-host reaction. At this time not only is the RES hyperactive, but intense cellular proliferation is found in the spleen and lymphoid follicles. Much later in the course of severe graft-versus-host disease, when the subject is grossly deficient in lymphoid tissue, increased susceptibility to some infections might be anticipated. Indeed, Vos and Van Bekkum (1959) have found that the mortality rate from severe graft-versus-host disease in mice could be reduced by continuous administration of antibiotics.

The increased resistance to pneumococcal infection described in this communication is almost certainly determined directly by efficient clearance of bacteria and their subsequent destruction by the hyperactive RES. Liver perfusion experiments have shown that normal Kupffer cells will phagocytose *D. pneumoniae* only when suspended in specific immune serum (Manwaring and Coe, 1916), whereas non-immune serum is completely devoid of opsonic activity and no phagocytosis can be detected (Wardlaw and Howard, 1959). However, the protection afforded against pneumococcal infection by bacterial endotoxin cannot be transferred by plasma or serum and is believed to be directly related to hyperactivity of the RES (Cooper and Stuart, 1962). Similarly, humoral opsonic

factors, although not excluded, seem unlikely to play a major role in the protection observed during the graft-versus-host reaction.

Attempts to improve the survival rate from salmonella infections in mice by non-specific means are generally unsuccessful. On the other hand, previous activation of the RES by various materials frequently prolongs survival of the animals by a few days (see review by Howard, 1961*c*). The slightly longer survival time of mice undergoing graft-versus-host reaction may well be a similar reflection of enhanced reticulo-endothelial activity. Paradoxically, although these mice are known to be hypersusceptible to bacterial endotoxin, they are slightly more resistant than normal to infection with an organism containing it. An identical situation arises during RE stimulation provoked by BCG and other *Mycobacterium* species (Howard, Biozzi, Halpern, Stiffel and Mouton, 1959; Biozzi, Stiffel, Halpern and Mouton, 1960).

Any generalization on results obtained with only two bacterial species would be premature. Obviously other types of infection as well as routes of injection require study. Nevertheless, the findings are sufficient to show that the increased susceptibility to bacterial infection reported in the later stages of the graft-versus-host reaction is not a necessary feature of the earlier, proliferative stage of the disease.

SUMMARY

Graft-versus-host reaction was induced in adult first generation (F1) hybrid mice by the injection of parental strain spleen cells. Twelve days later, when the phagocytic activity of the reticulo-endothelial system was enormously increased, the resistance of the mice to intravenously injected bacteria was examined. The mortality rate from a bacteraemic infection with *Diplococcus pneumoniae* was reduced from 80 per cent to nil. This effect was determined by rapid clearance of bacteria from the blood and their destruction in the liver and spleen. The mortality rate from *Salmonella typhimurium* infection was not improved, but the survival time of mice was slightly prolonged in the presence of graft-versus-host reaction.

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CHEMICAL PRODUCTION OF MYOCARDIAL NECROSIS WITH VASCULAR OCCLUSION

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Received for publication May 24, 1961

IT is known that in animals concurrent treatment with 9α-fluorocortisol and certain electrolytes gives rise to massive myocardial necroses that resemble true infarcts but are unaccompanied by lesions of the cardiac vessels (Selye, 1958*a*, 1961).

Previous studies have shown that a few minutes after the intravenous injection of a saccharated iron oxide preparation, iron-containing precipitates form intravascularly. Many of these lodge in the capillaries throughout the body, particularly in those of the lungs and kidneys (Cappell, 1929, 1930; Polson, 1928, 1929; Nissim, 1953*b*; Ellis, 1956).

The question arose whether treatment with 9α-fluorocortisol plus Na_2HPO_4 can predispose to the preferential precipitation of saccharated iron oxide in cardiac vessels.

MATERIALS AND METHODS

Eighty female rats of the Holtzman farm, with a mean initial body weight of 95 g. (range 90–100 g.) were divided into 8 equal groups and treated as indicated in Table I.

9α-fluorocortisol (F-COL) was injected subcutaneously as a microcrystal suspension at the daily dose of 1 mg. in 0·2 ml. of water. Na_2HPO_4 was administered by gavage at the dose of 1 mM in 2 ml. of water twice daily until the end of the experiment.

One ml. of a ferric oxide saccharate preparation (FeOS) containing 20 mg. of iron ("Proferrin", Sharpe and Dohme) was very slowly injected into the jugular vein, under light ether anaesthesia, on the second and fourth day of the experiment. Instead of FeOS, a 50 per cent sucrose solution (similar to that employed in the preparation of the FeOS) was given to the controls, all other experimental conditions being the same.

Throughout the experiment the rats were maintained on "Purina Laboratory Chow" and tap water. On the fifth day the experiment was terminated and all surviving animals were killed with chloroform.

The hearts, kidneys, and livers were first inspected by means of a binocular loupe at autopsy. Tissues were fixed in alcohol-formol (2 parts 10 per cent formalin, 8 parts absolute alcohol), embedded in paraffin, and sectioned at 6–7 μ. Some sections were stained with haematoxylin-phloxine or PAS (to show the general structure) or with the von Kóssa silver nitrate technique (for the histochemical demonstration of calcium), while duplicate sections were examined for iron, using Perl's stain with carmalum or neutral red counterstain. The mean grades of the myocardial lesions listed in Table I are based on the examination of the entire, freshly dissected heart with the binocular loupe. The severity of the changes was graded in terms of an arbitrary scale 0 to 3 (0 = no lesions, 1 = barely detectable lesions, 2 = moderate lesions, 3 = most severe lesions). The mean grades of these lesions and the mean final body weight (with the standard error) are listed in Table I.

TABLE I.—*Myocardial Necroses with Vascular Occlusion Produced with 9α-fluorocortisol and Sodium Phosphate Plus Saccharated Iron Oxide*

Group	Treatment	Final body weight	Cardiac lesions (scale: 0–3)
I	FeOS (Saccharated iron oxide)	95·5±2·30	0
II	F-COL (9α-fluorocortisol)	82·0±1·04	0
III	Na_2HPO_4	107·0±1·18	0
IV	Na_2HPO_4 + FeOS	100·7±0·20	0
V	Na_2HPO_4 + F-COL	78·0±1·50	0
VI	F-COL + FeOS	73·0±1·50	0·9±0·28
VII	F-COL + Na_2HPO_4 + FeOS	75·0±2·00	2·5±0·27
VIII	F-COL + Na_2HPO_4 + sucrose	76·0±2·00	0

RESULTS

By the end of the experiment, of the ten animals in Group VII, six had died, exhibiting macroscopically visible patches of myocardial necrosis as well as intense renal lesions.

Animals treated with F-COL showed a loss in body weight (15 g.), which was not influenced by administration of FeOS.

The general pattern of iron deposition in Group I was similar to that reported by other authors (Polson, 1928; Cappell, 1929, 1930; Nissim, 1953*a*). FeOS, like other colloidal suspensions, is taken up by the reticulo-endothelial system. After treatment with doses such as were used in this experiment, the hepatic parenchymal cells contain only fine granules giving a faint homogeneous Prussian-blue reaction. In the Kupffer cells, large granules of pigment obscure the nuclear detail and the cells appear as masses of iron granules. An excess of iron is also found in the spleen, where it is deposited principally around the Malpighian bodies.

In the kidney, smaller amounts of iron are seen in the proximal convoluted tubules and a few cells with granules are present in the glomeruli. Occasionally intravascular precipitates develop in the capillaries of the lungs and kidneys.

Some cardiac lesions did occur even when only F-COL + FeOS was administered (Group VI), but they were much more marked in animals given Na_2HPO_4 in addition (Group VII). Macroscopically, the necrosis was often seen to be distributed in parallel lines, so that the heart assumed a tigroid aspect (Fig. 1). Microscopically, the necrotic muscle fibres were occasionally found to be impregnated with calcium demonstrable with the von Kóssa stain. Many capillaries and medium-sized veins were occluded by Prussian-blue positive-granular matter. A number of the vessels so affected were situated in the necrotic areas (Fig. 2).

Microscopically, the liver showed a heavy and uniform accumulation of iron in the parenchymal and reticulo-endothelial cells. A number of necrotic cells were faintly and homogeneously stained with Prussian blue.

Macroscopically, the kidney presented an uneven surface covered with white patches (Fig. 3). Microscopical examination showed that the glomerular capillary endothelium contained a very heavy iron-deposition (Fig. 4); iron was also seen in the stroma and in some tubular cells, but to a lesser degree. In many glomeruli, masses of precipitate heavily laden with iron occluded one or more capillaries. At the corticomedullary junction there was calcification with subsequent desquamation of tubular epithelia. No abnormalities were seen in the basement membranes of the glomerular capillaries, in sections stained with PAS.

These lesions occurred only in animals receiving F-COL + FeOS, with or without Na_2HPO_4; neither macroscopical nor microscopical lesions were observed in the animals of Groups I, II, III, V, or VIII.

DISCUSSION

A number of procedures have been devised in our Institute for the production of myocardial infarction by direct interference with the blood supply of the heart (Selye, Bajusz, Grasso, and Mendell, 1960). It has been shown, furthermore, that cardiac infarction with organic obstruction of the coronary vessels can regularly be produced by humoral means. Doses of Na_2HPO_4 + methylchlorocortisol that cause only occasional microscopic foci of myocardial necroses in non-pretreated animals produce, in the dihydrotachysterol-pretreated rat, intense proliferative and eventually obstructive changes in the coronary tree, with macroscopically visible patches of necrosis throughout the heart (Selye, 1958*b*).

Similar obstructive lesions were observed also in rats treated with various other cardiotoxic agents (*e.g.*, vasopressin, noradrenaline, papain, cold) after sensitization with dihydrotachysterol (Selye and Bajusz, 1959).

The object of the present experiments was to show whether a brief treatment with F-COL + Na_2HPO_4 can create humoral changes capable of provoking precipitation of the iron preparation, thereby inducing vascular occlusion in the heart. This proved to be the case : when the animals were pretreated with F-COL alone or (even better) in combination with Na_2HPO_4, intracapillary precipitation following FeOS administration was markedly affected in that it occurred predominantly in necrotic areas of the heart. There was no histological alteration in the wall of the occluded vessels. The necrotic lesions were small but affected all parts of the myocardium ; they were not restricted to isolated large " infarctoid " patches characteristic of the phosphate-steroid cardiopathy (Selye, 1958*a*, 1961).

It may be concluded that corticoids (*e.g.*, F-COL), alone or in combination with Na_2HPO_4, can condition for intravascular precipitation of saccharated iron oxide in the heart, thereby causing thrombosis and occlusion of cardiac vessels.

SUMMARY

Rats pretreated with 9α-fluorocortisol (F-COL) plus Na_2HPO_4 regularly develop cardiac lesions, following intravenous administration of saccharated iron oxide.

EXPLANATION OF PLATES

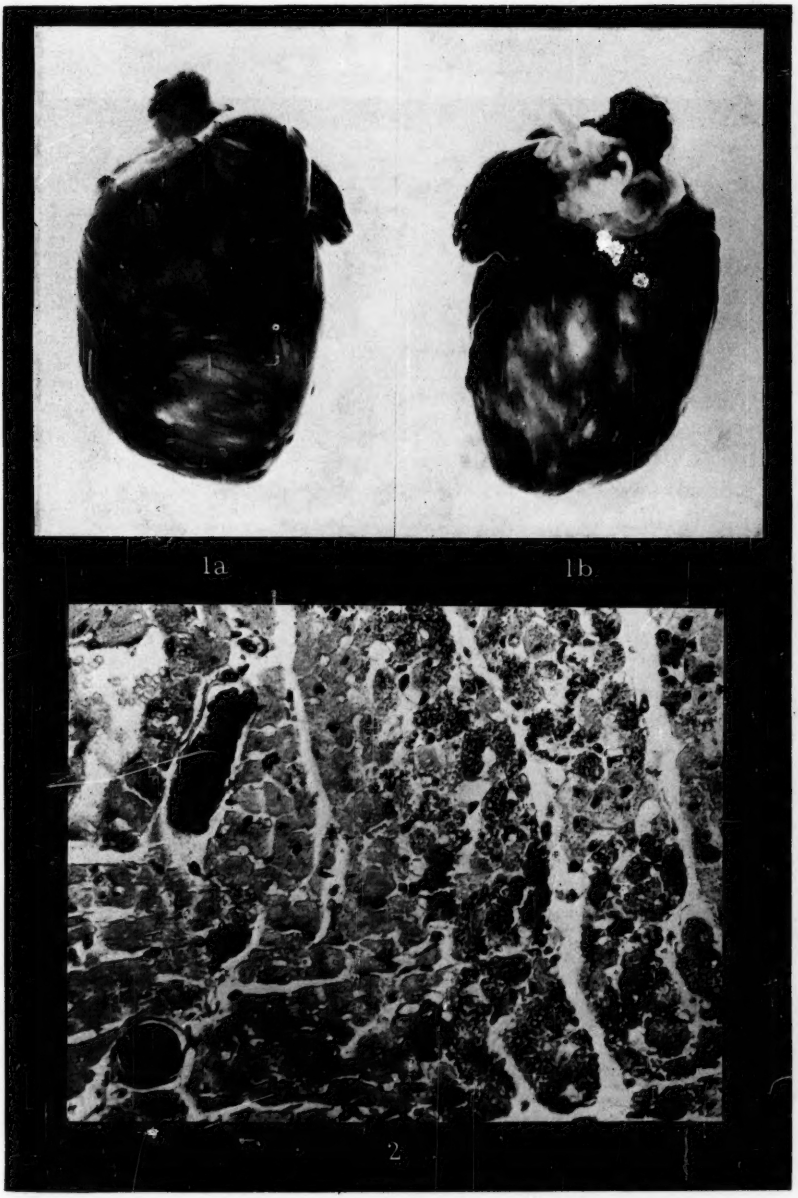
FIG. 1.—" a " anterior view and " b " posterior view of the heart. Characteristic distinct light streaks of myocardial necroses in an F-COL + Na_2HPO_4-conditioned rat treated with FeOS.

FIG. 2.—Complete obliteration of the lumen of some vessels of the heart by Prussian-blue-positive granular matter accompanied by necrotic muscle fibres impregnated with calcium, in rat given F-COL + Na_2HPO_4. Von Kóssa. ×360.

FIG. 3.—Kidneys of 2 rats treated with F-COL + Na_2HPO_4. " a " : not otherwise treated. " b " : injected with FeOS, showing intense nephrocalcinosis.

FIG. 4.—Heavy iron deposition in glomerular endothelium of the capillaries of rat treated with F-COL + Na_2HPO_4 + FeOS; some of the capillaries are completely occluded. Prussian blue × 300.

FIG. 5.—Glomerular lesions similar to those of Fig. 4 ; in addition, occlusion of one medium-sized vessel. Prussian blue. ×300.



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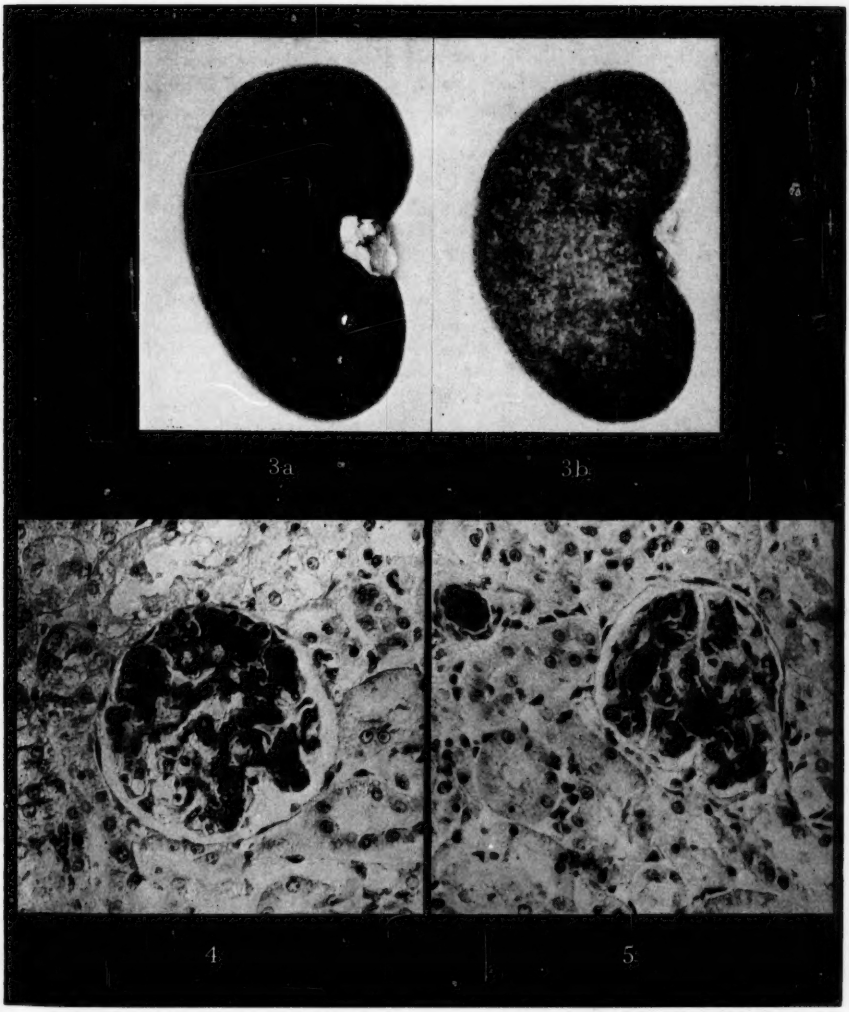

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4 5

This cardiopathy is characterized by " tigroid " streaks of myocardial necroses with thromboses in the capillaries and medium-sized veins of the heart.

Under these experimental conditions, the cardiac vessels appear to be selectively predisposed for the formation of clots.

This work was supported by the National Heart Institute Grant No. H-6182, U.S. Public Health Service ; The Dept. of Health and Welfare, Prov. of Quebec ; and The Gustavus and Louise Pfeiffer Research Foundation.

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FAT EMULSIONS AND FIBRINOLYSIS

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Received for publication June 2, 1961

THE finding of inhibition of *in vitro* fibrinolysis of lipaemic plasma clots as measured by a modification of Bidwell's technique (Bidwell, 1953) was described from this laboratory (Greig, 1956). Later work showed that cream and probably egg yolk meals resulted in this inhibition while vegetable oil meals did not. Studies on solvent-treated plasmas led to the suggestion that " the β lipoproteins and/or chylomicrons, depending on their composition " might be responsible for the inhibition observed (Greig and Runde, 1957).

The *in vitro* experiments to be described show that emulsions of various fats have interesting effects on fibrinolytic systems, in high concentrations inhibiting streptokinase induced human plasma clot lysis, and yet at the same time act as proactivator when studied by the fibrin plate technique (*i.e.* with streptokinase are able to induce lysis of unheated bovine fibrin). The emulsions have been shown to have an affinity for a protein with the properties of plasminogen and are able to adsorb it from serum. This affinity appears to be enhanced by the incorporation of phosphatide into the emulsion particles. A similar dual activity on the part of platelets has also been demonstrated. The relationship of these findings to previously reported ones will be discussed.

MATERIALS AND METHODS

Normal human plasma.—This was obtained from out-of-date citrated pooled blood bank blood through the courtesy of Dr. A. Zoutendyk. A single batch, preserved with 1/10,000 merthiolate and kept at + 4°, was used for all the experiments to be described.

Streptokinase.—This was the Lederle Laboratories' (American Cyanamid Co.) product Varidase. The material was dissolved in normal saline to give solutions of the desired concentration. The manufacturers' stated unitage (in Christensen units) was used.

Borate buffered saline.—This was prepared after Palitzsch (1915). The pH of the solution was 7·6 as measured with a glass electrode.

Bovine fibrinogen.—Armour and Co.'s bovine Cohn Fraction I was used without further purification. It was dissolved at a concentration of 0·4 per cent (w/v) in borate buffered saline.

Bovine thrombin—The product Thrombin Topical of Parke-Davis Limited was used. It was dissolved in borate buffered saline to give solutions of the desired concentration. The manufacturers' stated unitage (N.I.H. units) was used.

Phosphatide.—The alcohol insoluble fraction of soy-bean phosphatides, marketed by Associated Concentrates Inc., New York, as Inosithin, was used. It is stated to contain lecithin, cephalin and inositol phosphatide. It was emulsified in borate buffered saline for use.

Fat emulsions.—Two proprietary fat emulsions were used, Ediol, a coconut oil emulsion prepared by Schen-Labs, New York, and Lipomul, a product of Upjohn Co., Kalamazoo, Michigan. Ediol is a 50 per cent emulsion of coconut oil emulsified with polyoxyethylene sorbitan monostearate (2 per cent w/v). Lipomul is a 15 per cent (w/v) cotton-seed oil emulsion containing lecithin (1·29 per cent w/v) in addition to oxyethylene oxypropylene polymer (0·4 per cent w/v). These emulsions were diluted as required with borate buffered

saline. Natural cream was used after several washings with normal saline by gravity. Vigorous handling or centrifugation resulted in butter formation and was accordingly avoided.

Emulsions of other fats were prepared with the aid of a 2 per cent aqueous solution of Triton W. R. 1339 (Rohm and Haas Inc.). Triton is a polyoxyethylene ether. The emulsions were prepared by mixing appropriate volumes of 2 per cent Triton solution and the fat in an Atomix blender (M.S.E. Limited, London) for 5–10 min. at high speed. This usually resulted in a stable emulsion. In certain cases phosphatide (Inosithin) 1 per cent w/v was incorporated in the emulsions by adding the appropriate weight of Inosithin emulsified in saline, to the emulsion in the blender and mixing for a further 5–10 min.

Activated emulsions.—This signifies emulsions which have been incubated with normal human serum for 1 hr. in the proportion of 1 part of serum to 3 parts of emulsion and then the lipid particles washed repeatedly. The emulsion after incubation with serum was centrifuged for 60 min. at 15,000 rpm (R.C.F. = 17,000 *g*) in an M.S.E. refrigerated centrifuge at + 4°. The infranatant material was withdrawn with the aid of a long fine needle and syringe and the supernatant emulsion resuspended in 0·85 per cent (w/v) saline buffered at pH 7 with 0·001 M phosphate buffer containing 0·05 per cent sequestrene (di-sodium salt). Resuspension of the particles was effected with the aid of a syringe and No. 27 gauge hypodermic needle with repeated aspiration and expulsion through the needle. The process of washing and centrifugation was repeated up to 10 times.

Streptokinase-activated human plasma clot lysis

To 0·5 ml. of normal citrated human plasma in a 75 × 7 mm. I.D. tube, 0·5 ml. of the material under test was added, in controls 0·5 ml. of borate buffered saline was added, 0·1 ml. of a streptokinase solution was added and a stopwatch started. The tube was inverted to mix the contents. Exactly 10 sec. after the addition of the streptokinase, 0·1 ml. of a thrombin solution was added and the stop-watch reset to zero and a second stop-watch started. When the plasma clotted, the first stop-watch was stopped. The tube was then continuously observed for lysis. When the clot had entirely lysed, the second stop-watch was stopped. The time on the second stop-watch, less the clotting time of the plasma indicated on the first stop-watch, gave the lysis time of the clot.

The usual concentration of streptokinase used was 2000 Christensen units per ml., *i.e.* 200 units per test. This concentration gave a lysis time in the region of 100–200 sec. as a rule, but was not necessarily the concentration of streptokinase giving the shortest lysis time. Considerable variability from batch to batch of streptokinase and of nominally equivalent dilutions of the same batch was noted, however. The concentration of thrombin used was 25 N.I.H. units per ml., *i.e.* 2·5 units per tube. Within the range 25–150 units/ml. of thrombin there was no significant (at the 1 per cent level) difference in the plasma clot lysis time using 2000 units streptokinase per ml. (Table I). A concentration of 25 N.I H. units

TABLE I.—*Plasma Clot Lysis Times. Effect of Varying Thrombin Concentration. Streptokinase Concentration 2000 u/ml*

Thrombin concentration (units/ml.)	*n*	Clotting times (sec.)	Lysis times (sec.)	
25	5	20·4	170·0 ± 3·56	$P > 0·1$
50	5	19·6	167·6 ± 3·35	
100	5	12·8	188·8 ± 3·06	
150	5	12·0	176·4 ± 4·25	

Clotting times = Mean of 5 determinations.
Lysis times = Mean ± standard error of mean (5 determinations).

per ml. gave clotting times in control tubes of 20–45 sec. as a rule. Certain fats were found to greatly delay clotting, *e.g.* cream ; others appeared to enhance it, *e.g.* paraffin emulsion.

An interval between addition of streptokinase and addition of thrombin of up 60 sec. did not affect the lysis time found (Table II) ; it did, however, seriously affect the precision of the results (cf. the standard errors of the means). Accordingly, a standard interval as short as practicable, *i.e.* 10 sec., was chosen.

TABLE II.—*Plasma Clot Lysis Times. Effect of Varying Interval Between Addition of Streptokinase and Thrombin*

Interval between addition of streptokinase and thrombin (sec.)	Number of tests (n)	Clotting times (sec.)	Lysis times (sec.)
10	10	27·9	157·1±2·01
30	5	30·2	158·6±7·96
60	5	40·4	156·8±10·19

Clotting times = Mean of n determinations.
Lysis times = Mean ± standard error of mean (n determinations).

At least 5 estimations and often more were made for each material. The method was found to give very consistent results for a given set of reagents. The results are expressed in seconds ± the standard error of the mean. P is estimated by Student's t-test (Mainland, 1952).

Estimation of activator activity by Astrup and Alkjaersig's (1952) *unheated fibrin plate method*

Fibrin plates were prepared by clotting 12·5 ml. of 0·4 per cent bovine fibrinogen solution in a 9 cm. Petri dish with 2 units of bovine thrombin. The plates were stored at + 4° until used. The material to be tested was incubated with streptokinase for 15 min. at 37° then applied in 30 µl. amounts on to the surface of the fibrin. The plates were incubated for 16–18 hr. at 37°. As a rule 3 determinations were done on each 9 cm. Petri dish.

The area of the lysed zone gives a measure of the "activator" activity of the material. The area in mm.² is obtained by taking the product of 2 perpendicular diameters of the lysed zone. It was found convenient to remove the lysed fibrin with a fine Pasteur pipette in the case of the emulsions as their opacity made measurement difficult.

RESULTS

Streptokinase induced human plasma clot lysis

Cream.—Table III shows the effect of various concentrations of cream on the lysis time of plasma clots. Marked inhibition is found with undiluted (10 per cent) cream and is still found in a 1/5 dilution of this cream. The marked prolongation of clotting time is also seen in Table III. As these experiments were

TABLE III.—*Plasma Clot Lysis Times. Effect of Cream*

Material	n	Clotting times (sec.)	Lysis times (sec.)	P
Cream (undiluted)	5	89·4	290·2±13·63	<0·001
Cream 1/1	5	54·0	196·2±7·22	<0·01>0·001
Cream 1/5	5	35·2	186·0±8·24	<0·02>0·01
Controls	10	27·9	157·1±2·01	—

Clotting times = Mean of 5 determinations.
Lysis times = Means ± standard error of mean (5 determinations).

carried out with commercially obtained 10 per cent cream containing other material beside emulsified butter fat, an experiment was carried out using an artificial emulsion of clarified butter. Fresh creamery butter was melted, washed and clarified and then used to prepare a 50 per cent (w/v) emulsion with 2 per cent Triton W.R. 1339. When this emulsion was tested, it was also found to cause inhibition of streptokinase induced plasma clot lysis (Table IV). The degree of

TABLE IV.—*Plasma Clot Lysis Times. Effect of a 50 per cent W/V Emulsion of Butter Fat prepared with 2 per cent Triton*

Material	*n*	Clotting times	Lysis times	*P*
Butter fat emulsion (undiluted)	10	36·7	159·2±1·45	<0·01
Butter fat emulsion 1/5	8	37·8	144·7±3·57	=0·05
Butter fat emulsion 1/10	10	39·1	136·0±2·14	>0·1
Controls	10	35·6	135·8±2·63	—

Clotting times = Mean of *n* determinations.
Lysis times = Mean ± standard error of mean (*n* determinations.)

inhibition obtained with these artificial emulsions was not as great as that obtained with cream. The reason for this is not known. It may be that components responsible for the inhibition are lost to some extent in the process of butter making or, alternatively, that the physical properties of the artificial emulsion, or the emulsifying agent used, affect the result.

TABLE V.—*Plasma Clot Lysis Times. Effect of Triglyceride Emulsions*

Material	*n*	Clotting times	Lysis times	*P*
a. Ediol—				
Undiluted	5	52·7	170·2±3·36	<0·001
1/10	5	33·4	140·6±3·71	<0·05>0·01
1/100	5	29·7	126·8±3·17	>0·1
1/1000	5	29·5	129·0±2·18	>0·1
Controls	9	28·3	132·0±1·46	—
b. Lipomul—				
Undiluted	5	24·6	123·6±1·28	<0·001
1/10	5	28·7	126·6±4·19	<0·05>0·01
1/100	5	29·8	117·8±1·74	<0·001
1/1000	5	29·8	119·0±2·79	<0·001
$1/10^6$	5	29·4	126·4±2·40	<0·02>0·01
Controls	14	28·3	133·3±1·23	—

Clotting times = Mean of *n* determinations.
Lysis times = Mean ± standard error of mean (*n* determinations).

Triglyceride emulsions.—In Table V studies with 2 triglyceride emulsions, Ediol and Lipomul, are shown. Ediol is found to produce considerable inhibition, undiluted and in a 1/10 dilution while "Lipomul" on the other hand does not show inhibition at any concentration but a significant activation.

Lipomul contains 1·4 per cent w/v lecithin. To determine if the presence of phosphatides would alter the inhibitory action of Ediol, 1 per cent w/v of Inosithin was incorporated into a 1/1 dilution of Ediol in saline. Table VI shows that

TABLE VI.—*Plasma Clot Lysis Times. Effect of Phosphatides on Ediol Inhibition*

Material	*n*	Clotting times	Lysis times	*P*
1/1Ediol	7	56·8	195·5±3·74	<0·001
1/1 Ediol + 1 per cent phosphatide	6	56·2	205·0±4·28	<0·001
Controls	6	44·2	145·0±1·70	—

Comparing Ediol and Ediol + phosphatide, $P > 0·1$.
Clotting times = Mean of *n* determinations.
Lysis times = Mean ± standard error of mean (*n* determinations).

there was no significant difference in the lysis time in presence of 1/1 Ediol alone or 1/1 Ediol containing Inosithin, both being equally inhibitory compared with the controls.

It is to be noted that Ediol is a 50 per cent (v/v) oil-water emulsion when undiluted, while Lipomul is a 15 per cent (v/v) oil-water emulsion when undiluted. An attempt was made to concentrate Lipomul by high speed centrifugation and resuspension of the creamy layer in a smaller volume of saline. An approximately $2\frac{1}{4}$ times concentration of the material was achieved but it was still not inhibitory.

Liquid paraffin emulsion.—A 25 per cent (v/v) emulsion of liquid paraffin B.P. prepared with 2 per cent Triton W.R.-1339 had the effect of significantly reducing both the clotting time of the substrate plasma and the clot lysis time. The incorporation of 1 per cent w/v Inosithin into the emulsion had no effect on this reduction of clot lysis time (Table VII).

TABLE VII.—*Plasma Clot Lysis Times. Effect of a Mineral Oil Emulsion, With and Without Phosphatide*

Material	n	Clotting times	Lysis times	P
Paraffin emulsion	6	20·4	101·1±2·44	<0·001
Paraffin emulsion + 1 per cent phosphatide	5	20·9	102·8±1·53	<0·001
Controls	5	32·9	128·8±3·59	—

Clotting times = Mean of n determinations.
Lysis times = Mean ± standard error of mean (n determinations).

Platelets.—A suspension of human platelets, washed 6 times and containing 5×10^6 platelets per c.mm. was found to induce marked inhibition of streptokinase induced plasma clot lysis (Table VIII).

TABLE VIII.—*Plasma Clot Lysis Times. Effect of a Platelet Suspension*

	n	Clotting times	Lysis times	P
With platelets	5	44·8	271·0±4·69	<0·001
Controls	5	40·6	161·8±6·78	—

Clotting times = Mean of n determinations.
Lysis times = Mean ± standard error of mean (n determinations).

TABLE IX.—*Activator Activity on Unheated Fibrin Plates*

Material	Area of lysis (mm.²)
Lipomul emulsion	100
"Activated" Lipomul emulsion washed 5 ×	315
Ediol emulsion	95
"Activated" Ediol emulsion washed 5 ×	150
Cream "10 per cent" washed 10 ×	64
"Activated" cream washed 10 ×	132
Liquid paraffin, 25 per cent emulsion	60
"Activated" paraffin emulsion washed 5 ×	94
Platelets, 5×10^6 per mm.², washed 8 ×	236
Saline	Nil

The results are the mean of 3 determinations. The materials were all incubated with 1000 u streptokinase/ml. before application to the fibrin plate. "Activated" signifies incubation of the emulsion with serum.

TABLE X.—*Activator Activity on Unheated Fibrin Plates*

State of emulsion	Serum treated		Saline treated	
	Ediol	Lipomul	Ediol	Lipomul
Unwashed	480	315	64	95
1st wash	342	270	68	108
2nd ,,	174	203	73	105
3rd ,,	144	156	64	86
4th ,,	105	150	64	81
5th ,,	105	138	64	100
6th ,,	105	127	80	95

This table shows the area of lysis in mm.^{2} (mean of 3 tests) occurring in 17 hr. on unheated fibrin plates as a result of application of the emulsion after incubation with 1000 u. streptokinase per ml. for 15 min. The aliquots removed at each wash were adjusted to the same optical density prior to incubation with streptokinase. The emulsion concentrations on account of this were much lower than those in Table IX.

Activator activity of emulsions as measured on fibrin plates

Table IX shows the ability possessed by some of the emulsions tested to act as activator in conjunction with streptokinase in the lysis of bovine fibrin when studied by the unheated fibrin plate method. It is observed that the untreated emulsions possess this activity (Greig, unpublished), but pre-incubation of the emulsions with serum leads to higher activator activities even after multiple washings. Lipomul appears to be more active than Ediol in this respect, possibly due to its lecithin content. Table X shows the effect of multiple washings on these emulsions.

Platelets were found to possess marked activator activity (Table IX). Multiple washings in physiological saline containing 0·05 per cent sequestrene, even up to 20 times, does not remove this property from the platelets. However, saline in which such platelets have been incubated for 30 min. at 37°, shows activator activity with streptokinase, although freed from particulate material by centrifugation. This indicates that the " proactivator " in platelets is soluble and this observation should provide an opportunity for its study and identification.

DISCUSSION

Our previous studies, as outlined in the introduction, led to the conclusion that the blood lipids exerted influences, at times inhibitory, but also at times apparently activatory, on fibrinolysis as studied *in vitro*. Our subsequent studies, of which this is one, have been endeavours to discover how the blood lipids influence fibrinolysis.

As these effects were demonstrated during lipaemia, it was natural to investigate the chylomicrons. These have been found, even after many washings, to have marked activator activity (*i.e.* the ability, with streptokinase, to convert bovine plasminogen to plasmin), but have no measurable proteolytic activity with streptokinase, against casein (Greig and Cornelius, 1960).

Korn (1955) has shown that artificial triglyceride emulsions, *e.g.* of coconut oil (Ediol) acquire properties after incubation with human serum in respect of the heparin-activated lipoprotein lipase system which the untreated emulsions do not possess. It was thought well to study such emulsions in fibrinolytic test systems.

An unexpected finding was that oil emulsions, untreated with serum, were able,

with streptokinase, to induce lysis of bovine fibrin clots on the unheated fibrin plate technique. This study is reported elsewhere (Greig, unpublished). In this paper the activator activity of the serum-treated (" activated ") emulsions, which is much higher than that of the non-serum-treated emulsions, is reported.

However, parallel studies by the streptokinase activated human plasma clot lysis technique showed that some of the emulsions, but not all, are inhibitory to lysis.

Our explanation of these findings is that chylomicrons and lipid emulsions have affinity for plasminogen, and bind it. In the bound form, plasminogen can still (if it be human plasminogen) act as activator with streptokinase to bovine or other animal plasminogens, although it does not show proteolytic activity, with streptokinase, against casein. This affinity of triglyceride emulsions (*e.g.* Ediol) for human plasminogen (purified by the method of Kline (1953)) can be readily verified experimentally. On the other hand, no affinity of emulsions for plasmin or for streptokinase can be shown. (Greig, unpublished.)

The different results with the two techniques used in this study can therefore be explained. Under the conditions used in the streptokinase activated clot lysis method, the lysis is largely due to activation of the plasma plasminogen to plasmin prior to clotting. If an emulsion able to bind the plasminogen is present, lysis will be delayed. On the other hand, with the fibrin plate technique, the plasminogen present is associated with the fibrin, and the reaction time permitted is very long. Lipid-associated human plasminogen under these circumstances can act as activator of the bovine plasminogen associated with the bovine fibrin of the plate. The fact that untreated emulsions can act as weak activators with streptokinase, suggests that the lipid-associated form of bovine plasminogen is also susceptible to activation by streptokinase.

The finding that phosphatide containing emulsions appear to have greater affinity for plasminogen is of interest, especially in view of the activator activity of platelets. However, affinity and inhibitory binding do not run in parallel, as Lipomul, although having as high affinity as Ediol (Table X) does not produce inhibition in the plasma clot lysis test, whereas Ediol does. These observations may have some bearing on the different effects (inhibition or activation) on *in vitro* fibrinolysis of lipaemic plasma clots seen as a result of feeding different fats (Greig and Runde, 1957). It also suggests that the polar phospholipid moiety, believed to lie at the periphery of the chylomicron and to be largely responsible for its stability, may be of prime importance in plasminogen binding.

Platelets have previously been shown to be inhibitory to fibrinolysis (Johnson and Schneider, 1953 ; Stefanini and Murphy, 1956 ; Hume, 1958 ; Hougie and Ayres, 1960), but their ability to act as activator with streptokinase does not seem to have been described. It seems of especial interest that they should exhibit the same properties as oil emulsions. Platelets have been shown to contain a clottable protein, probably identical with fibrinogen (Ware, Fahey and Seegers, 1948 ; Johnson and Schneider, 1953 ; Seligmann, Goudemand, Janin, Bernard and Grabar, 1957 ; Vazquez and Lewis, 1960 ; Silber, Benitez, Eveland, Ackroyd and Dunne, 1960). Seligman *et al.*, Vazquez and Lewis, and Silber *et al.*, were able to identify fibrinogen only as the plasma protein found in association with the platelet, but Salman (1960) claims to have identified albumin, haptoglobin, macroglobulins and γ globulin by immunochemical methods. Of these, the γ globulin might be of greatest interest, as plasminogen in the " proactivator "

form appears to migrate electrophoretically as a γ globulin, whereas plasminogen in serum migrates as a β globulin. (Greig, unpublished.)

These *in vitro* observations suggest some ideas in relation to thrombolysis, *i.e.* the dissolution of thrombi *in vivo*.

The adhesion of platelets to fibrin strands and threads during clotting is well known, forming masses of agglutinated platelets, especially at the intersection of the fibres. This is generally thought to be concerned with clot retraction although it is of interest that some have thought that fibrinolysis may be involved in the contraction of the fibrin clot. (Roskam, 1927 ; Ferguson and Erickson, 1939 ; Ferguson, 1960). *In vivo* the " white head " of a thrombus is largely platelet material.

It has also been shown (Bang and Cliffton, 1960) that clots formed *in vivo* during alimentary lipaemia in dogs, contain concentrations of chylomicrons in close association with the fibrin threads.

Thus, both these components, platelets and chylomicrons, which we believe possess activator activity on account of their affinity for plasminogen, are found in close association with fibrin threads deposited *in vivo*. Whether the fibrin has an affinity for these particles because of their content of plasminogen or vice versa must await further studies. The former seems more likely, but the basis of the affinity of fibrinogen for plasminogen is not known. (Sherry, Fletcher and Alkjaersig, 1959).

Two consequences may be surmised. If the chylomicrons on account of their composition effect inhibitory binding of the plasminogen, then it is possible to see how clots formed *in vivo* during, for example, cream lipaemia, will be persistent owing to inhibition of fibrinolysis (Bang and Cliffton, 1960). It also affords an alternative explanation to the infiltration hypothesis for the presence of lipids in the atheromatous lesion. The fibrin deposit, prevented from lysing by the inhibitory attached chylomicrons, may on incorporation, as postulated by Duguid (1946), carry the lipid in with it.

Thanks are due to the Director and Deputy Director of the South African Institute for Medical Research for generous facilities for carrying out this work.

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ACTIVE AND PASSIVE IMMUNITY TO TRANSPLANTATION OF FOREIGN BONE MARROW IN LETHALLY IRRADIATED MICE

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Received for publication June 3, 1961

WORK in several laboratories has shown beyond doubt that lethally irradiated mice may be induced to recover by the intravenous implantation of haematopoietic cells from normal mice, and that their recovery is due to repopulation of the host by the injected cells and their progeny (Ford, Hamerton, Barnes and Loutit, 1956). Barnes and Loutit (1954) showed that restoration could not be effected by cells of an allogeneic, that is genetically foreign (Gorer, Loutit and Micklem, 1961) mouse against whose antigens the host had previously been immunized. In other words, the state of immunity had not been abolished by the radiation. Three interpretations of this were possible. Either the injected bone marrow cells were destroyed by humoral antibody circulating in the serum of the host; or they were the object of a cellular immune response mounted by sensitized radioresistant cells; or they were affected by both kinds of response. The immune response to solid homografts of skin is mediated primarily by cells (Billingham, Brent and Medawar, 1954; Billingham and Brent, 1956), and it is not clear that humoral antibody, though it may be formed (Amos, Gorer, Mikulska, Billingham and Sparrow, 1954; Micklem and Brown, 1961), plays any part in the rejection of such solid grafts. Against certain tumours (notably lymphomas), on the other hand, humoral antibody is undoubtedly active both *in vitro* and *in vivo* (Gorer, 1958).

The experiments reported in this paper were undertaken to extend information on the bone marrow treatment of pre-immunized irradiated mice, and to discover whether destruction of injected bone marrow cells in these mice was due to a cell-mediated or a humoral reaction. While the experiments were in progress, Gorer and Boyse (1959), Santos, Cole and Garver (1959), and very recently Garver and Cole (1961) reported work showing that immunity to injected marrow suspensions could be passively transferred by means of specific antisera. Our results with some, but not all, strain combinations confirm those of the above workers, and also show that the amount of antiserum needed may be very small.

MATERIALS AND METHODS

Stocks

All the mice used in these experiments were bred at Harwell. They belonged to the strains CBA, A, C57BL and C3H, all of which are inbred by strict sib-mating with frequent reselection of lines. All mice were 3–5 months old at the time of irradiation.

Immunization

Mice were immunized against iso-antigens by one of three methods.

Method 1.—Three injections of approximately 10^7 adult spleen cells suspended in saline were given at intervals of a fortnight—the first intravenous, the second intraperitoneal and the third subcutaneous. Recipients were irradiated or bled for serum 10–14 days after the third injection.

Method 2.—Two successive orthotopic grafts of foreign skin were applied, the second 20 days after the first. Serum was collected 36 days after the second graft was completely shed.

Method 3.—Approximately 10^7 adult spleen cells were injected intraperitoneally. Five to 6 days later the recipients were grafted orthotopically with skin from the same donor strain. Serum was collected 14 days after application of the graft.

Some serum pools prepared in CBA mice against A or C57BL iso-antigens by Methods 1 and 3 were titrated for haemagglutinins by the dextran method of Gorer and Mikulska (1954) and found to have titres of 1/16–1/128, the lower titres being produced by Method 1. Sera prepared by Method 2 probably had titres around 1/64 (Micklem and Brown, 1961). These antisera were cytotoxic in the presence of complement to the corresponding lymphoid cells when tested *in vitro* by a modification of the method of Gorer and O'Gorman (1956).

Irradiation

Mice were X-irradiated in boxes of 5 by the standard technique of this laboratory (Corp, 1957). The dose was 1007 rads ± 3 per cent (250 kV.; 14 ma.; H.V.L. 1·2 mm. Cu.) which is 99 per cent lethal to our CBA, C3H and A mice within 30 days, nearly all the mortality being within 14 days.

Suspensions for intravenous therapy

The method of preparing these has been described by Bridges, Loutit and Micklem (1960).

EXPERIMENTAL RESULTS

Experiment 1

Two hundred and twenty CBA ♀, 80 A ♀, 20 C3H ♂, and 27 C57BL ♀ mice were used as hosts in this experiment. The aim was to confirm that pre-immunization of mice against allogeneic mouse tissue antigens (in this case spleen) prevented their recovery after irradiation and treatment with bone marrow of the same foreign strain. In addition we tested the capacity of C57BL ♂ antigens to immunize C57BL ♀ hosts against a subsequent injection of C57BL ♂ bone marrow. In most strain combinations, three experimental groups were run concurrently :—

1. Allogeneic bone marrow or bone marrow and spleen→irradiated pre-immunized hosts.
2. Allogeneic bone marrow→irradiated non-immune hosts.
3. Saline only→irradiated non-immune hosts.

The third group was omitted when C3H and C57BL hosts were used. Method 1 was used for immunization.

Table I shows the survival times seen in the four strain-combinations tested. These results show that except in the C3H→CBA strain combination all mice in Group 1 died within 14 days of irradiation—*i.e.* within the normal period of death from acute radiation-induced aplastic anaemia. Gross autopsy showed no difference between Group 1 and Group 3, in which mortality was 100 per cent within 2 weeks. All mice had small lymph-nodes and spleen, minute thymus, and pale liver and kidneys. The bone marrow was haemorrhagic and gastro-intestinal haemorrhage was common, though not universal. Histological examination of 14 animals in Group 1 confirmed the basic lesion as aplasia of the bone

TABLE I.—*Experiment* 1 : *Mortality of Normal and Actively Iso-immunized Mice after Lethal Whole-body X-irradiation and Treatment with Foreign Haematopoietic Tissue*

Hosts	Donor material	Number of mice	Number dead in ⩽ 14 days	Number dead in 15–30 days	Survivors > 30 days
CBA immune to A	A bone marrow	25	25	0	0
CBA immune to A	A bone marrow and spleen	25	25	0	0
Normal CBA	A bone marrow	25	2	1	22
Normal CBA	Saline only	25	25	0	0
A immune to CBA	CBA bone marrow	25	25	0	0
A immune to CBA	CBA bone marrow and spleen	25	25	0	0
Normal A	CBA bone marrow	25	1	0	24
Normal A	Saline only	5	5	0	0
CBA immune to C3H	C3H bone marrow	25	18	4	3
CBA immune to C3H	C3H bone marrow and spleen	25	25	0	0
Normal CBA	C3H bone marrow	25	1	3	21
Normal CBA	Saline only	25	25	0	0
C3H immune to CBA	CBA bone marrow	10	10	0	0
Normal C3H	CBA bone marrow	10	0	0	10
C57BL ♀ immune to C57BL ♂	C57BL ♂ bone marrow	13	9	0	4
Normal C57BL ♀	C57BL ♂ bone marrow	14	2	0	12

marrow. A large proportion of Group 1 died also in the C3H→CBA and C57BL ♂ →C57BL ♀ combinations.

In Group 2 deaths within 14 days were the exception : almost all animals were successfully restored by the injected bone marrow and died later at times which varied with the strain combination. Such late deaths are now generally considered to be initiated by an immune reaction by the grafted tissue against the host, although in the CBA→ A and CBA→ C3H strain combinations such a reaction, if it takes place, is feeble. (An account of secondary mortality in weakly and strongly reacting strain combinations is in preparation).

These results confirmed those of Barnes and Loutit (1954). Repopulation and restoration of irradiated mice with foreign bone marrow was indeed prevented when the hosts had been immunized against the appropriate iso-antigens. The death of a majority of immunized mice in the C3H→ CBA and C57BL ♂→C57BL ♀ combinations showed that the effect was not solely dependent on differences at the H-2 histocompatibility locus, and that even the antigens associated with the Y-chromosome were able to immunize C57 ♀ hosts effectively.

Experiment 2

This experiment was designed to show whether repopulation could be prevented by the injection of a fairly large amount (0·4 ml.) of immune serum 1–2hr. before lethal irradiation. Fifteen CBA ♂ mice were injected intravenously before irradiation with 0·4 ml. of pooled serum from CBA mice immunized by Method 1

against A-strain spleen cells. Fifteen control mice received 0·4 ml. of pooled normal CBA serum. All were intravenously injected ½–4 hr. after irradiation with 2–5 × 10^6 nucleated cells from the bone marrow of normal A donors. Mortality is shown in Table II. The animals which received immune serum all died within 14 days from aplasia of the bone marrow, while the controls died between 30 and 120 days after irradiation from " secondary disease ".

TABLE II.—*Experiment* 2 : *Mortality of CBA Mice Lethally Irradiated and then Injected Intravenously with A-strain Bone Marrow*

Hosts	Number of mice	Number dead in ≤ 14 days	Number dead in 15–30 days	Survivors > 30 days
Passively immunized before irradiation*	15	15	0	0
Controls†	15	0	0	15

* Injected intravenously 1–2 hr. before irradiation with 0·4 ml. undiluted CBA anti-A serum.
† Injected intravenously 1–2 hr. before irradiation with 0·4 ml. undiluted normal CBA serum.

This experiment suggested that serum antibodies were alone sufficient to bring about the rejection of grafted marrow ; sensitized cells were not necessary.

The next step was to discover how small a quantity of serum could successfully transfer immunity of this kind. A-strain mice were in short supply, so C57BL donors were used in the following experiments.

Experiment 3

One hundred and twenty CBA ♂ mice were divided into 4 groups of 30. Half the mice in each group received serum from CBA mice immunized against C57BL spleen cells by Method 1 ; this was given intravenously ½–2 hr. before irradiation. The other half received normal CBA serum. The experiment was timed so that it was not necessary to store sera for longer than overnight after bleeding of the donors. The maximum period of storage was 18 hr. at 0–4°. All mice were injected intravenously with 2–5 × 10^6 nucleated cells from normal C57BL bone marrow ½–4 hr. after X-irradiation with 1007 rads. The quantity of serum given to each group was as follows :—

Group 1. 0·4 ml. neat serum.
Group 2. 0·4 ml. serum diluted 1/10 in saline.
Group 3. 0·4 ml. serum diluted 1/100 in saline.
Group 4. 0·4 ml. serum diluted 1/1000 in saline.

The results are shown in Table III.

The minimum effective dose of antiserum was somewhere between 0·004 ml. and 0·0004 ml. It would presumably vary with the number of foreign cells injected, and this was not accurately measured.

In another experiment similar results were obtained with serum from mice immunized with skin grafts (Method 2), although the minimum effective dose was rather larger (0·04 ml.–0·004 ml.).

Experiment 4

The strains C57BL and CBA differ genetically at the important H–2 histocompatibility locus (Snell 1958 ; Gorer, personal communication). The strains

TABLE III.—*Experiment* 3: *Mortality of CBA Mice Injected Intravenously with* 0·4 *ml. of Various Dilutions of CBA Anti-C57BL Serum*, then Lethally Irradiated and Injected with C57BL Bone Marrow. Controls Received Normal CBA Serum*

Group	Number of mice	Dilution of serum	Number dead in < 14 days	Number dead in 15–30 days	Survivors > 30 days
1 Exptl.	15	Neat	15	0	0
Control	15	Neat	4	5	6
2 Exptl.	15	1/10	15	0	0
Control	15	1/10	3	2	10
3 Exptl.	15	1/100	15	0	0
Control	15	1/100	3	6	6
4 Exptl.	15	1/1000	0	2	13
Control	15	1/1000	0	4	11

* Donors given 3 fortnightly injections of 10^7 C57BL spleen cells.

C3H and CBA, on the other hand, both have the phenotype H–2^k and give relatively weak immune reactions against each other. Results in Experiment 1 showed that CBA mice immunized against C3H spleen rejected C3H bone marrow administered after lethal irradiation. Could this immunity to weak iso-antigens be passively transferred, as in Experiment 3?

Sixty CBA ♂ mice were divided into 2 groups. Thirty were injected intravenously $\frac{1}{2}$–2 hr. before irradiation with pooled antiserum to C3H spleen (prepared by Method 1). The other 30 received pooled normal CBA serum. The volume injected was 0·4 ml. of either neat serum or serum diluted 1/10 in saline.

The results are shown in Table IV.

TABLE IV.—*Experiment* 4: *Mortality of CBA Mice Injected Intravenously with* 0·4 *ml. of Neat or Diluted CBA Anti-C3H Serum, then Lethally Irradiated and Injected with C3H Bone Marrow*

Method of immunizing serum donors	Number of mice	Dilution of serum	Number dead in < 14 days	Number dead in 15–30 days	Survivors > 30 days
Method 1*	15	Neat	1	1	13
Nil (control)	15	Neat	2	1	12
Method 1*	15	1/10	1	0	14
Nil (control)	15	1/10	1	0	14
Method 3†	10	Neat	0	0	15
Nil (control)	10	Neat	0	0	15

* Three fortnightly injections of 10^7 C3H spleen cells.
† Intraperitoneal injection of 10^7 C3H spleen cells. Grafted 6 days later with C3H skin.

The mortality in experimental and control mice was indistinguishable. Any immunity to C3H antigens transferred in 0·4 ml. of serum was insufficient to prevent repopulation by 2–5 million C3H bone marrow cells. This result was confirmed with another pool of antiserum, prepared by Method 3. In addition, experiments were performed in which C3H bone marrow cells were incubated with antiserum *in vitro* before being injected into lethally irradiated mice. Again

there was no evidence that the serum was lethal to the cells; only a small percentage of the cells took up trypan blue or nigrosin after incubation, and all the irradiated mice survived beyond 14 days (Table V).

TABLE V.—*Experiment* 4: *Viability of C3H Bone Marrow Cells after Incubation with CBA Anti-C3H Serum*, Measured by Exclusion of Nigrosin and Capacity to Restore Lethally Irradiated CBA Mice. Controls were Incubated with Normal CBA Serum*

Pretreatment of donor bone marrow cells	Number of cells injected per mouse	Percent staining after incubation	Number of mice	Survivors > 30 days
1 hr. at room temp. in 1 : 1 serum : Tyrodes, without added complement				
Exptl.	About 2×10^6	—†	10	10
Control	About 2×10^6	—†	10	10
Overnight at 0–4° then 1 hr. at room temp. in 1 : 2 : 1 serum : Tyrodes : guinea-pig complement				
Exptl.	$2{\cdot}25\times10^6$	25	10	9
Control	$2{\cdot}44\times10^6$	26	10	10
2 hr. at 37° in 1 : 2 : 1 serum : Tyrodes : guinea-pig complement				
Exptl.	$2{\cdot}46\times10^6$	15	10	10
Control	$2{\cdot}46\times10^6$	13	10	9

* Prepared by Method 3.
† Not counted.

Experiment 5

It seemed almost certain that immunity to bone marrow transplants persisted only so long as the specific antibodies continued in sufficient concentration in the circulation. To confirm this supposition, it was necessary to use a pool of antiserum of known potency, irradiate the serum-injected hosts after various intervals, and inject them with a fixed number of bone marrow cells. The expected duration of passive immunity could be roughly estimated by reference to the reported half-life of mouse γ-globulin—about 2 days according to Dixon, Talmage, Maurer and Deichmiller (1952) and 4½ days according to Humphrey and Fahey (1961).

A pool of immune serum (CBA anti-C57BL) was prepared by Method 3 and stored at −15° in several ampoules until needed. All the serum used in the experiment was thus frozen once only and thawed immediately before use. Its potency was estimated as follows. Various dilutions were injected intravenously into CBA ♂ mice and 24–26 hr. later the mice were irradiated and injected intravenously with $2{\cdot}0\times10^6$ bone marrow cells from 4-month-old C57BL ♀ donors. This system differed in two respects from that employed in the previous experiments. Firstly, there was a longer interval (24–28 hr.) between the injections of serum and bone marrow. Secondly, the number of bone marrow cells injected was more closely controlled.

The results of this *in vivo* " titration " are shown in Table VI. The smallest volume of antiserum which was completely effective in preventing repopulation was 0·01 ml. Some effect was seen after the injection of 0·001 ml., but not, paradoxically, of 0·004 ml.

For the second part of the experiment, 0·4 ml. of a 1/10 dilution of antiserum (*i.e.* four times the minimum completely effective dose) was injected intravenously

TABLE VI.—*Experiment* 5 : *Mortality of CBA ♂ Mice Injected Intravenously with 0·4 ml. of Various Dilutions of a Single Pool of CBA Anti-C57BL Serum, then Lethally Irradiated the Following Day and Injected with* $2{\cdot}0 \times 10^6$ *C57BL Bone Marrow Cells*

Dilution of serum injected		Number of mice	Number dead in ⩽ 14 days	Number dead in 15–30 days	Survivors > 30 days
1/10	E*	10	10	0	0
1/10	C†	10	0	0	10
1/40	E	10	10	0	0
1/40	C	10	1	0	9
1/100	E	10	0	0	10
1/100	C	10	0	2	8
1/400	E	10	4	2	4
1/400	C	10	0	0	10
1/1000	E	10	2	1	7
1/1000	C	10	0	1	9

* Experimental : immune serum injected.
† Control : normal serum injected.

into 80 CBA ♂ mice, 80 controls receiving a like quantity of normal CBA serum by the same route. Groups of 10 experimental and 10 control mice were irradiated with 1007 rads and injected with $2{\cdot}0 \times 10^6$ C57BL♀ bone marrow cells on the 3rd, 7th, 14th, 21st, 28th, 42nd, 56th and 70th days after the injection of serum. The mortality among these animals is shown in Table VII.

If we take as an index of complete immunity the prevention of repopulation in 90 per cent of the injected mice, then complete immunity was present on the 3rd and 7th days after the injection of antiserum. Immunity was partial on the 14th day, but on the 21st day it was again almost complete—9 out of 10 mice dying by the 15th day after irradiation. A similar paradox was noted above in the results of the first part of the experiment, where 0·001 ml. of antiserum appeared to transfer more immunity than 0·004 ml. On the 28th and subsequent days after the injection of antiserum no detectable immunity remained : mortality in the experimental and control groups was indistinguishable.

DISCUSSION

" Transplantation immunity " by means of antisera

The present experiments parallel in some respects those of Kaliss (1958), Amos and Day (1957), and Gorer and Kaliss (1959). These workers implanted tumour cells in mice which had circulating humoral antibody against the tumour antigens. The antibody was either actively produced by the host in response to immunization with killed tumour preparations, or passively transferred by way of serum from immunized donors. The remarkable differences in the response given by various tumours to various doses of anti-serum have attracted much attention. Some tumours (notably leukoses) are destroyed or inhibited by the action of the specific antiserum ; others not merely survive, but grow with increased speed (" enhancement ") ; yet others may show a dual response, being

TABLE VII.—*Experiment* 5: *Mortality of CBA ♂ Mice Injected Intravenously with* 0·4 *ml. of a Single Pool of CBA Anti-*C57BL *Serum (Diluted* 1/10); *then, After Various Intervals, Lethally Irradiated and Injected with* $2{\cdot}0 \times 10^6$ *C57BL Bone Marrow Cells*

Interval between serum injection and irradiation (days)		Number of mice	Number dead in ⩽ 14 days‡	Number dead in 15–30 days‡	Survivors > 30 days‡
3	E*	10	10	0	0
	C†	10	0	1	9
7	E	10	9	1	0
	C	10	2	0	7
14	E	10	4	1	5
	C	10	0	0	10
21	E	10	7	2§	1
	C	10	1	1	8
28	E	10	0	3	7
	C	10	0	1	9
42	E	10	2	1	7
	C	10	0	0	10
56	E	10	0	1	9
	C	8	0	0	8
70	E	10	0	1	9
	C	10	1	0	9

* Experimental: immune serum injected.
† Control: normal serum injected.
‡ Post-irradiation.
§ Both dead on 15th day.

inhibited by high doses of antiserum and "enhanced" by low doses (Gorer and Kaliss, 1959).

It is possible that normal tissue homografts show a similar range of responses. Billingham, Brent and Medawar (1956) found a small degree of enhancement of skin homografts in mice pretreated with killed tissues. Parkes (1958) found considerable enhancement of orthotopic ovarian homografts in similarly treated rats. In neither case was the enhancing effect passively transferred by injecting serum from a pre-treated animal into a normal host. Kaliss (1958) reported failure to produce enhancement of normal mouse skin homografts in his laboratory.

Our own experiments demonstrate that, as with the leukoses of Amos and Day (1957) and Gorer and Kaliss (1959), so with normal bone marrow suspensions, there is destruction or inhibition of the implant in the presence of specific antiserum. It seems probable that this destruction or inhibition takes place almost immediately following intravenous injection of the cells, by a cytotoxic mechanism resembling that which is demonstrable *in vitro* by the method of Gorer and O'Gorman (1956). It is scarcely possible that sensitized host cells played any part in Experiments 2–5, and there is no need to suppose that they did so in Experiment 1. The failure of attempts to prevent growth of C3H cells by means of CBA anti-

C3H serum, despite the active immunity induced against C3H in Experiment 1, may be due simply to failure to produce a potent enough serum. The immunization schedules employed in these experiments certainly result in much lower titres of haemagglutinin than are obtainable by other methods (Brown and Micklem, unpublished). On the other hand this may be one instance in which the intervention of sensitized cells or cell-bound antibody is necessary.

The presence of antiserum results in a state of transplantation immunity to injected bone marrow suspensions. This does not necessarily mean that there is a state of transplantation immunity in a general sense to all tissues carrying the appropriate antigens. Billingham and Brent (1956) have given strong evidence that humoral antibodies alone are ineffective against skin, and the same is probably true of most solid tumour grafts.

Duration of passively transferred iso-immunity

The results of Experiment 5, which was intended to show the duration of passive immunity, are impossible to interpret with certainty owing to their apparent inconsistency. There can be little doubt that the failure of 0·004 ml. of serum to confer immunity to bone marrow injected the following day, while 0·001 ml. of the same serum was partially effective, was due to some imperfection of the test system. One possible variable is the proportion of effective repopulating cells in the bone marrow inoculum. The apparent resurgence of the immunity conferred by 0·04 ml. of serum between the 14th and 21st days after injection may reasonably be ascribed to similar causes. Nevertheless it is difficult to make the results fit with the half-life of antibody quoted by Dixon *et al.*, (1952)—1·9 days. The possibility must be borne in mind that a portion of the injected antibody may become attached (perhaps temporarily) to cells of the host, like the " cytophilic antibody " of Boyden and Sorkin (1960), and thus escape metabolism at the normal speed. Alternatively the γ-globulin of iso-antibody may be metabolized at a slower rate than that of the antibody measured by Dixon *et al.* Recent work (Humphrey and Fahey, 1961) shows this to be true of normal γ-globulin, the half-life of which may be as high as $4\frac{1}{2}$ days. A half-life around this figure fits our results better than one of 2 days.

Effect on survival of subliminal levels of antibody

It seemed possible that levels of antibody just insufficient to cause acute death of the irradiated host through failure of repopulation might have some effect on longer term survival. By killing a proportion of the injected cells they might reduce, or delay the onset of, an immune reaction by the graft against the host; or they might act in the opposite way, by stimulating mitosis and so causing such a reaction to develop more rapidly. (The former effect would be analogous to that reported by Křen, Veselý, Frenzl and Štark (1960). They found that the runting syndrome initiated by injecting foreign spleen cells into newborn rats could be inhibited by the previous injection of iso-antiserum to blood of the donor. The erythropoietic elements of the spleen were not destroyed, since the recipients remained blood chimaeras, but runt disease was reduced or prevented.) Close comparison of the time of onset, the duration and the outcome of " secondary disease " in animals with low antibody levels and in controls failed to reveal any differences. Effects such as those postulated, if they existed, were too small to be detected.

SUMMARY

Mice from 3 highly inbred strains were actively immunized against the iso-antigens of other inbred strains. In addition C57BL female mice were actively immunized against the iso-antigens, determined by the Y-chromosome, of C57BL males. They were then given a lethal dose of X-rays followed by an injection of bone marrow from donors of the strain against which they had been immunized. In these circumstances the bone marrow therapy, which saved non-immune control mice from acute radiation death, was ineffective.

In two strain combinations, in which there was considerable antigenic disparity between donor and host, immunity could be transferred by injecting serum from immunized donors intravenously. As little as 0·004 ml. of immune serum was effective. It was concluded that serum antibodies were alone able to mediate immunity to transplanted bone marrow.

In a third strain combination, where donor and host had the same antigens determined by the H-2 histocompatibility locus, passive immunity was not demonstrated. In this case, effective immunity may have required the presence of sensitized cells.

It is a pleasure to acknowledge the technical assistance of Mr. J. W. Hayton, Mrs. J. Sansom, and Mr. P. H. Embling.

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THE SEROLOGICAL DETERMINATION OF HISTOCOMPATIBILITY ACTIVITY

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Received for publication June 19, 1961

AGREEMENT has not yet been reached about the relationship between histocompatibility, transplantation and enhancing antigens, but the same genetic system underlies all these activities and the trend of opinion is that at least the specificity of the antigens is likely to be the same (see *e.g.* Snell, 1957 ; Brent, Medawar and Ruszkiewicz, 1961).

For following the increase of activity during the isolation and purification of substances carrying these properties, the enhancement of tumour growth in incompatible hosts (Kandutsch and Reinert-Wenck, 1957 ; Kandutsch, 1960) or the hastening of skin graft rejection (Billingham, Brent and Medawar, 1956) give excellent specificity, but lack sensitivity. While no characteristic component of the substances can be assessed by chemical estimation in conjunction with one of these methods, a serological test seems the most promising.

The haemagglutination test of Gorer and Mikulska (1954, 1959), using dextran and normal human serum (NHS), is a simple and reliable method for detecting " incomplete " antibodies of the H2 system which dominates these reactions. We describe below some examples of how this test can be inhibited to measure the activity of cells and insoluble materials, and also of soluble preparations carrying histocompatibility specificity.

The information provided by these tests allows one not only to follow the trend of activity on fractionation, but also to make the best choice of starting material. We have been led in this way to use the products of mouse ascites tumours, which can provide the necessary amounts of material for chemical work.

MATERIALS AND METHODS

Mouse strains

Inbred lines of C3H/He, C57BL, BALB/c and Strong A have been used ; breeding stock was provided by the Laboratory Animals Centre (M.R.C. Carshalton).

Ascites tumours

The following tumours were used : Landschütz (" Lan ", non-specific), BP8 and 6C3HED (specific for C3H mice), Sa-1 (A mice), EL4 (C57BL mice) and CL2 (BALB/c mice).

Antibody production

Mice were immunized with fresh specific tumour cells (separated from red cells by centrifuging through a sucrose gradient) from different strains by 4 subcutaneous injections at weekly intervals. Four days before the last immunizing injection, non-specific or isologous tumour cells were injected intraperitoneally to induce ascitic fluid, which was recovered 12 days later and used as source of antibody. To avoid confusion, this immune ascitic fluid will be referred to hereinafter as antiserum. The antisera were stored freeze-dried and samples

dissolved in distilled water at 40 mg./ml. as required ; this is the approximate concentration of total solids in mouse ascitic fluid.

Haemagglutination tests

The dextran-NHS system is well known and has been clearly described (Gorer and Mikulska, 1954). Briefly, antiserum is diluted in 1 per cent dextran, and red cells (at 1 per cent in a 1 : 2 dilution of inactivated NHS, which has previously been absorbed with similar red cells) are added to each tube. Agglutination is read microscopically after incubation at 37° for 90 min.

Measurement by absorption.—For intact cells, or materials which can be reasonably well dispersed in aqueous suspension, equal amounts of material were added to all tubes of a series of antiserum dilutions, before the addition of red cells, and a measure of activity recorded as the difference between the haemagglutination titre thus obtained and the titre given by the unabsorbed antiserum.

The antiserum dilutions were made in 1 per cent dextran (8 drops) and one drop of suspension under test added. The mixtures were incubated for 1 hr. at 37° and centrifuged if necessary. To 2 drops of each supernatant solution were added 2 drops of 1 per cent red cell suspension (red cells in 1 : 2 NHS which had previously been inactivated at 56° for 30 min. and absorbed with similar red cells) and incubated for 90 min. at 37°. Haemagglutination was then read microscopically without delay.

Measurement by dilution inhibition.—Soluble materials are more easily tested by dilution in antiserum fixed at a small number of haemagglutinating doses, but again in this system haemagglutination does not occur in the absence of dextran and NHS.

A suitable range of serum dilutions is judged by direct agglutination of appropriate red cells. Maximum sensitivity is then arranged by testing several dilutions of serum in this range against doubling dilutions of a specific inhibitor. The antiserum is subsequently used at a dilution where maximum inhibition is obtained while the serum control gives a safely positive score. A negative control with the maximum concentration of inhibitor used, but omitting antiserum, is also essential.

To the diluted serum in 1 per cent dextran (2 drops) is added 2 drops of the diluted inhibitor. After incubation at 37° for 60 min., red cells are added (2 drops of 1 per cent red cells in 1 : 2 NHS previously inactivated and absorbed) and incubation at 37° continued for 90 min. Haemagglutination is then read microscopically without delay.

Preparation of soluble antigens

Materials carrying histocompatibility specificity were prepared from various ascites tumour cells or ascitic fluids. The methods used are described elsewhere (Davies, 1961).

RESULTS AND DISCUSSION

The direct agglutination of various mouse red cells by different mouse anti-mouse sera is shown in Table I ; all the combinations where agglutination occurs are ones expected to show H2 differences, as indicated by the antigens entered in the table in parentheses, although only the series C to K have been taken into account. In only 3 instances is there no agglutination where it might have been expected ; this is presumably due to insufficient immunization.

If different H2 specificities are carried by different molecules, polyspecific antisera would confuse the results of any fractionation procedure, and since there is no justification, as yet, for assuming that the specificities are carried by one molecule, then monospecific antisera, or antisera with the least possible range of antibodies would be most desirable. Considering the H2 antigens C to K only, monospecific systems do appear in Table I (C, D^b, D^k, E, E^d, F) where red cells do not carry all of the specifications corresponding to the antibodies in the particular serum. Other monospecific systems can be obtained by using various hybrids for immunization, *e.g.* C57BL × BALB/c F1 mice immunized with St. A

TABLE I.—*Agglutination of Mouse Red Cells by Various Mouse Anti-mouse Sera**

Agglutination of red cells from	Antisera from	Immunizing antigen: CL2/BALB (CDE^dFk)	BP8/C3H (CD^kEfK)	EL4/C57 (cD^bEFk)
C3H/He (CD^kEfK)	"A" (CDEFK)	†0 (—)	256 (D^k)	0 (—)
	BALB/c (CDE^dFk)	—	4096 (D^kEK)	1024 (E)
	C3H/He (CD^kEfK)	0 (—)	—	0 (—)
	C57BL. (cD^bEFk)	0 (C)	2048 (CD^kK)	—
BALB/c (CDE^dFk)	"A" (CDEFK)	128 (E^d)	0 (—)	0 (—)
	BALB/c (CDE^dFk)	—	0 (—)	0 (—)
	C3H/He (CD^kEfK)	2048 (DE^dF)	—	128 (F)
	C57BL. (cD^bEFk)	256 (CDE^d)	64 (C)	—
C57BL (cD^bEFk)	"A" (CDEFK)	0 (—)	0 (—)	256 (D^b)
	BALB/c (CDE^dFk)	—	0 (E)	1024 (D^bE)
	C3H/He (CD^kEfK)	0 (F)	—	512 (D^bF)
	C57BL. (cD^bEFk)	0 (—)	0 (—)	—

* Haemagglutination induced with dextran and normal human serum.
† Dilution of maximum haemagglutination titre, with antigens expected to react in parentheses.

mouse cells provide an H2-K serum suitable for following the purification of C3H material carrying H2-K specificity. Co-isogenic pairs of strains would provide even better systems.

The absorptive capacity of fresh BP8 tumour cells is shown in Table II, and a satisfactory relationship can be seen between increasing dilution of serum and of the cell suspension used for absorption. Freeze-dried cells retain their histocompatibility specificity and make a suitable standard which can be included in all tests. It is then possible to compare the activity of different cells, and, *e.g.* to follow the elution of active material from cells. It will be seen that tumour cells from mice not having H2-D^k, E, or K are quite inactive in this system (*e.g.* CL2/BALB) while Sa-1/"A" and 6C3HED/C3H are less active than BP8. The non-specific tumour Lan, grown in C3H mice, is quite inactive. The well known

TABLE II.—*Absorption of Histocompatibility Haemagglutinins**

Cells, mg./ml., used for absorption	Antiserum dilution: 4	8	16	32	64	128	256	512	1024
Nil (control)	†4	4	4	4	3	2	2	1	—
No dextran-NHS	—	—	—	—	—	—	—	—	—
BP8 (D^kEK) 10	3	2	1	—	—	—	—	—	—
5	4	3	2	1	—	—	—	—	—
2·5	4	4	3	3	2	—	—	—	—
1·25	4	4	4	3	2	1	—	—	—
0·6	4	4	4	4	3	2	1	—	—
0·3	4	4	4	4	4	3	2	1	—
LAN (H2-nil) 10	4	4	4	3	3	3	2	1	—
CL2 (DE^dk) 10	4	4	4	4	3	2	2	+	—
6ED (D^kEK) 10	4	3	3	2	1	—	—	—	—
Sa-1 (EK) 10	3	3	2	1	—	—	—	—	—
"L" cells (H2-K) 10	3	2	2	1	—	—	—	—	—
HeLa cells 10	4	4	4	4	3	2	2	+	—

* Using H2-D^kEK antiserum and C3H (D^kEK) red cells, in the dextran-normal human serum system.
† Degree of agglutination from 4 = maximum to + = minimum.

"L" strain mouse fibroblast tissue culture derived originally from C3H mice (Earle, 1943) retains its H2 character after nearly 20 years in culture, while HeLa cells are, of course, inactive.

Using different antisera, other specificities can be measured equally well, *e.g.* CL2/BALB, and EL4/C57 give specific inhibition in their own systems.

The quantity of material required for testing is small, *e.g.* at a level of 10 mg./ml. it amounts to only about 300 μg. per tube. Insoluble cell products can generally be sufficiently well dispersed by some means for testing, but very coarse suspensions can give only a qualitative answer.

The absorption test revealed that ascitic fluids, from which all cells had been removed, carried specific activity in soluble form. This led to the use of a more usual kind of dilution inhibition, an example of which is shown in Table III.

TABLE III.—*Inhibition of H2 Haemagglutination by Soluble Histocompatibility Specific Substances*

Nature of inhibiting antigen	Concentration of inhibitor added (μg./ml.)										
	4000	2000	1000	500	250	125	62	31	16	8	4
(BALB anti-C3H serum at 1 : 150, C3H red cells ; detection of H2-D^{k}EK)											
BP8 ascitic fluid .	—	*2	3	4	4	3	4	4	4	3	4
H2-D^{k}EfK . .	—	—	—	—	—	—	—	—	—	+	3
H2-DE^{d}Fk . .	3	3	4	3	4	4	4	3	4	4	4
H2-D^{b}EFk . .	—	—	—	3	4	4	3	4	4	3	4
H2-nil† . .	3	4	4	3	4	3	4	4	3	4	4
(C3H anti-BALB serum at 1 : 150, BALB red cells ; detection of H2-DE^{d}F)											
H2-D^{k}EfK . .	1	3	3	3	3	4	3	4	3	4	
H2-DE^{d}Fk . .	—	—	—	—	—	—	+	1	2	4	
H2-D^{b}EFk . .	—	—	2	2	3	4	4	3	4	4	
(BALB anti-C57 serum at 1 : 50, C57 red cells ; detection of H2-D^{b}E)											
H2-D^{k}EfK . .	—	—	—	—	—	—	1	2	3	4	
H2-DE^{d}Fk . .	3	4	3	3	4	4	3	4	4	3	
H2-D^{b}EFk . .	—	—	—	—	—	—	—	—	1	3	

* Degree of agglutination from 4 = maximum to + = minimum.
† Analogous product from a non-specific tumour fluid.

In this case also, comparisons can be made with a preparation chosen as a standard which can be included in each test. It will be seen that the most active H2-K preparation shown is some 500 times as potent as the starting material (BP8 ascitic fluid), but gives no inhibition in C3H anti-CL2/BALB serum ; it cross reacts in BALB/c anti-EL4/C57 serum due to H2-E. The purified product of BALB/c mice reacts well in its own system (H2-DE^{d}F) but gives no inhibition in either of the other systems shown, where no overlapping specificity occurs. On the contrary, the product of C57BL mice, which inhibits very strongly in its own system (H2-D^{b}E), also inhibits to a lesser degree in the other systems due to H2-E and H2-F.

Using the method of mixed agglutination (Coombs, Bedford and Rouillard, 1956), specific (BP8) and non-specific (Lan) tumour cells grown in C3H mice were tested with an appropriate antiserum. Positive mixed agglutination, with C3H red cells as indicator, was obtained with BP8 cells when these were used in 1 : 2

NHS, and antiserum (immune ascitic fluid diluted 1 : 5) diluted in 1 per cent dextran. Under these conditions Lan cells gave a negative result. BP8 cells were also negative when non-immune ascitic fluid was used, or when NHS or dextran was omitted from the system in which antiserum was included. This test does not readily lend itself to measurement of antigen or antibody, but it reveals that histocompatibility specificity is present on the surface of the tumour cells though not, of course, whether similar material is also present inside the cells.

The highest titre mouse anti-mouse sera have not given a line on agar diffusion plates when tested against the most active H2 antigens so far prepared, but using rabbit anti-BP8/C3H tumour cell sera a single line is found (tested as described by Crumpton and Davies, 1956). This is not an H2 line, but one corresponding to a substance not detected in normal mouse serum, but present in the structural component of mouse cells (Haughton, unpublished). The rabbit antiserum used in this case contains antibodies against a large number of normal mouse serum components and against several additional antigens confined to mouse cells.

SUMMARY

Histocompatibility antibodies can be detected readily in immune ascitic fluid induced after mouse anti-mouse immunization with ascites tumour cells.

Using the dextran-normal human serum system, the specificity carried by cells and insoluble materials can be detected and its potency measured by an absorption test.

The potency of soluble histocompatibility antigens can be measured by dilution inhibition of haemagglutination in the dextran-normal human serum system.

Mixed agglutination shows the presence of H2 specificity on the surfaces of ascites tumour cells.

It has not been possible to detect a line representing histocompatibility antigen-antibody by agar diffusion.

We are indebted to Professor A. Haddow, Chester-Beatty Research Institute ; the late Dr. P. A. Gorer, Guy's Hospital Medical School and Dr. J. Craigie, Imperial Cancer Research Fund, Mill Hill, for various mouse ascites tumours. We have our colleague Dr. W. Boyle to thank for carrying out the mixed agglutination tests, Dr. S. Pirt for samples of " L " cells and HeLa cells, and Dr. Paterson for breeding the mice which have been used.

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HISTOPATHOLOGICAL STUDY OF MUSCLE LESIONS PRODUCED BY PARAPHENYLENEDIAMINE IN RATS*

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Received for publication June 19, 1961

THE toxic properties of paraphenylenediamine (PPD) have been the object of several reports both in man and laboratory animals (Sidi, 1954; Rajka, 1952; Meissner, 1919). PPD given parenterally to rabbits causes an acute oedematous reaction of the head and neck, presumably as a result of increased capillary permeability and modification of the structure of ground substance (Tainter and Hanzlik, 1924; Halpern and Briot, 1954; Johnston, 1928). Typical vascular and connective tissue changes have also been described in rats following repeated cutaneous applications of the dimethylamino derivative of PPD (Geschickter, Athanasiadou and O'Malley, 1958). This derivative as well as other methyl substitutes was found to produce also in rats degenerative lesions in the central nervous system, lymphatic organs, pancreas, ovaries, skeletal and cardiac muscles (Jasmin, 1961). The present report concerns a histological study performed with a view to characterizing degenerative and regenerative changes that occur in striated muscles. An evaluation of the morphology of certain enzymes was also made.

MATERIALS AND METHODS

One hundred female Sprague Dawley rats averaging 115 g. body weight were used. They were fed Purina Fox Chow and tap water *ad libitum*. Dimethyl-PPD hydrochloride was injected subcutaneously as an aqueous solution at a dose of 2 mg. twice daily in a volume of 0·2 ml. To ensure that the preparation administered was of optimal and constant potency, solutions were prepared freshly before each treatment. Under such experimental conditions, muscle lesions develop in less than 4 days. To produce more rapid and severe lesions, the treatment, at the same dose level, may be administered thrice daily, but the majority of animals then soon develop fatal convulsions.

Except in experiments dealing with enzyme histochemistry, the rats were killed with chloroform. They were left untouched for a minimum period of 30 min. to prevent muscle contracture. In most cases, the gastrocnemius muscle and the heart were used for histological study. These organs were fixed in Susa sublimate solution. Sections were stained with haematoxylin-phloxine-saffron, Masson's trichrome and periodic acid-Schiff techniques. For the purpose of enzyme histochemistry the muscles were mounted on chucks and rapidly quenched in liquid air. They were stored in a sub-zero cabinet at $-70°$ and cut the following day on a cold microtome at $-20°$ and at 10μ thickness. The methods used for demonstration of enzyme activity are fully described in Pearse's book of histochemistry (Pearse, 1960).

RESULTS

Histogenesis of the lesions

A series of 60 rats received dimethyl-PPD for periods varying between 1–20 days. In order to follow the progression of muscle lesions, we killed a minimum of

* Supported by a research grant from the Muscular Dystrophy Association of Canada.

2 rats each day during the course of the treatment and the gastrocnemius was taken for histological studies.

On the first day, there was diffuse swelling of muscle fibres, some of which already had an homogeneous appearance. The interstitial exudative reaction was very conspicuous ; few inflammatory cells, mainly polymorphonuclear leucocytes, were present and could be seen only at this early stage (Fig. 1*a*).

On the second and the third day, the interfibrous oedema was prominent and the diameter of muscle fibres was extremely variable. The nuclei were increased in number and size and their arrangement was irregular. Several fibres acquired a granular aspect and lost their striation. A typical feature indicative of the severity of the degenerative process was the formation of contraction clots (Fig. 2*a*). But at this particular phase, coagulation necrosis with subsequent fragmentation and disintegration of the muscle fibres was the more characteristic feature (Fig. 1*b*). Such injured fibre segments act as foreign bodies giving rise to a histiocytic activity with proliferation of myophages (Fig. 1*c*). The isolated muscle fragment eventually undergoes phagocytosis. A similar reaction even took place around what seemed to be a relatively unaffected portion of the muscle fibres as judged by the presence of double striations. As the phagocytic cells proliferated, there was progressive disappearance of the exudative reaction.

From the fourth to the tenth day, while the animals were continuously under treatment, all previously described changes were liable to be found, plus some degree of regenerative activity. A number of sequestered muscle fibres remained surrounded by macrophages that eventually transform into multinucleated foreign-body giant cells (Fig. 1*d*). The latter were sometimes large enough to engulf the complete muscular fragment in their cytoplasm and to digest it progressively. As the sequestered muscle degenerated and became granular it acquired a typical brownish discoloration when stained with haematoxylin-phloxine-saffron (Fig. 2*b*). This material shrank and soon disappeared.

In spite of the widespread nature of these lesions it should be pointed out that no muscular dysfunction was apparent in such animals, presumably because the degenerative process alternated between fibres in such a way that there was no impediment to the activity of the muscle. Furthermore, the absence of extensive connective tissue fibrotic reaction helped to preserve the anatomical continuity of the muscle. Collagenization, when present, followed the longitudinal pattern of the muscle fibres.

After 10 *days*, the lesions became more and more discrete ; this finding suggests that a state of refractoriness develops progressively. Except for a slight interfascicular oedema and a relative increase in the number of nuclei, no change was demonstrable in spite of continuation of the treatment.

Lesions in the myocardium could be demonstrated after 3 days' administration of dimethyl-PPD. They appeared grossly as patchy discoloured areas mostly distributed on the lateral and posterior surfaces of the heart. A few foci could be found proximal to the epicardium, but more often they were located in the subendocardial region. Histologically, these foci are characterized by a proliferation of mononucleated cells, some of which resemble Anitschkow's myocytes. In all probability these cells derive from degenerated muscle fibres, but further studies will be made to ascertain this point. Unlike with lesions of the skeletal muscle, there are several mitotic figures ; however, no sign of regeneration can be observed. Also noteworthy is the fact that the degenerating foci became sclerotic.

Study of the regenerative activity

To further investigate the muscle lesions during the regenerative phase, a series of 30 animals treated with PPD during 4 days, were killed each day thereafter in groups of 3 ; the gastrocnemius muscle was taken for histology. The lesions observed on the third day following interruption of the treatment were particularly relevant. Some degenerative changes that corresponded approximately to those recorded on the seventh day were found, but here, they were more attenuated. Evidence of regeneration consisted in multiple areas of muscle fibres of various calibres and different degrees of basophilia (Fig. 3). This was accompanied by marked proliferation of both marginal and central nuclei within the fibres, which were usually arranged in rows even in the gracile basophilic fibres. The latter finding corresponds to the phenomenon designated by Durante as embryonic type of regeneration (Durante, 1902). However, some apparently unaffected fibres could also exhibit central nuclear rowing. The regenerative activity paralleled the severity of damage inflicted on the muscle fibres. By the 10th day, as a rule, there was complete restoration of the muscle, which structurally presented a normal appearance with no evidence of fibrosis or fatty tissue replacement.

Enzyme histochemistry

Ten rats treated with dimethyl-PPD during a period of 4 days were used for the demonstration of enzyme activity in the diseased muscles. Among the oxida-

EXPLANATION OF PLATES

FIG. 1.—Histogenesis of muscle lesions caused by dimethyl-PPD. All sections stained with haematoxylin-phloxin-saffron. ×60.

(*a*) Interstitial oedema with few polymorphonuclear leucocytes. Muscle fibres show little evidence of injury.

(*b*) Degeneration and rupture of muscle fibres. The exudative reaction is optimal.

(*c*) Proliferation of macrophages that tend to separate muscle segments.

(*d*) Sequestration of muscle segments by multinucleated giant cells. Note the alternation of degenerated fibres with relatively normal ones.

FIG. 2.—(*a*) Formation of a contraction clot in a muscle fibre undergoing granular degeneration. Note the integrity of the neighbouring fibres. Masson's trichrome. ×600.

(*b*) Sequestered muscle segments and phagocytosis by multinucleated giant cells. Note that striations are still present in the upper small segment. Haematoxylin-phloxin-saffron. ×240.

FIG. 3.—Regeneration of muscle fibres 3 days after discontinuation of treatment with dimethyl-PPD. All sections stained with haematoxylin-phloxin-saffron.

(*a*) Variation in size and stainability of fibres. Nuclei proliferate within the fibres both centrally and peripherally. ×75.

(*b*) Higher magnification of a basophilic regenerating fibre. ×300.

(*c*) Polymorphism and disorientation of nuclei. Note prominent nucleoli. ×300.

(*d*) Typical chain arrangement of nuclei within the regenerating fibre. ×300.

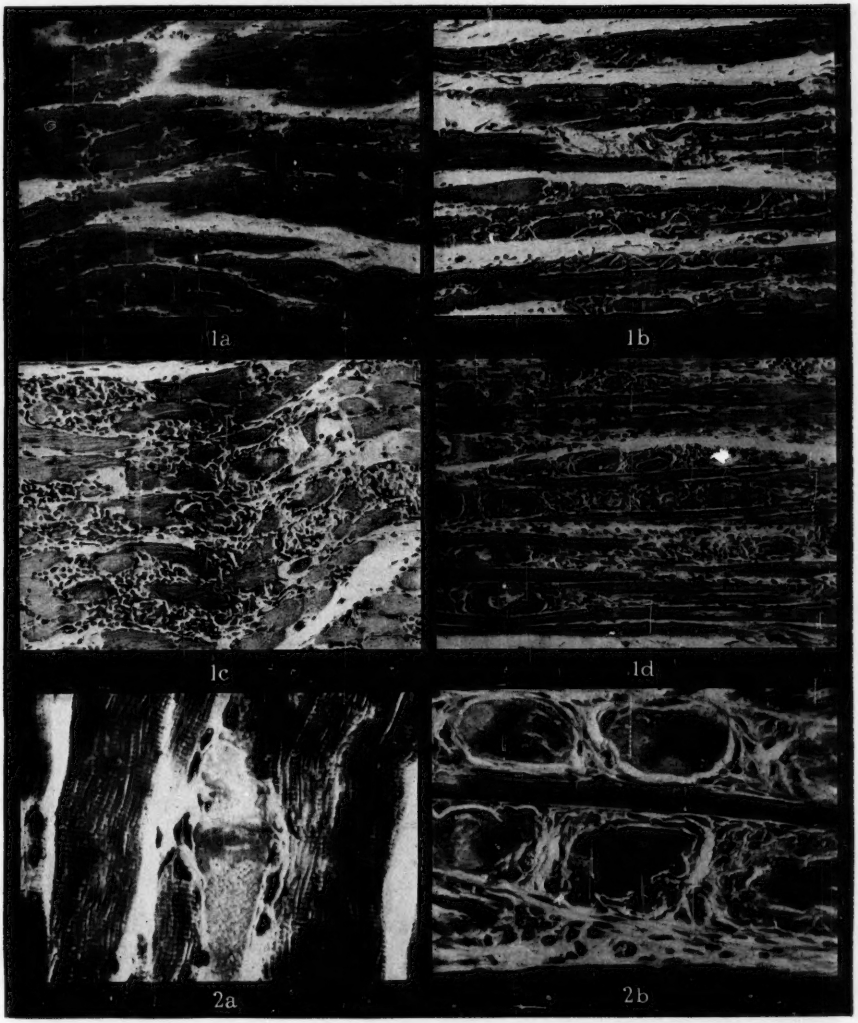
FIG. 4.—Changes in enzyme morphology in the thigh musculature after dimethyl-PPD treatment. ×190.

(*a*) Succinate dehydrogenase, in cross-section of a normal muscle. Note the subsarcolemmal localization of enzyme and difference in activity between small red fibres and large white fibres.

(*b*) After treatment with dimethyl-PPD, note the reduced activity of succinate dehydrogenase proportional to damage to fibres. There is a reduction in the calibre of fibres and a dispersion of the enzyme.

(*c*) Glucosan phosphorylase, in cross-section of a normal muscle. Note that the activity of the enzyme is higher in large white fibres.

(*d*) After treatment with dimethyl-PPD, note the reduced activity in phosphorylase in all type of fibres, in proportion to the degree of injury.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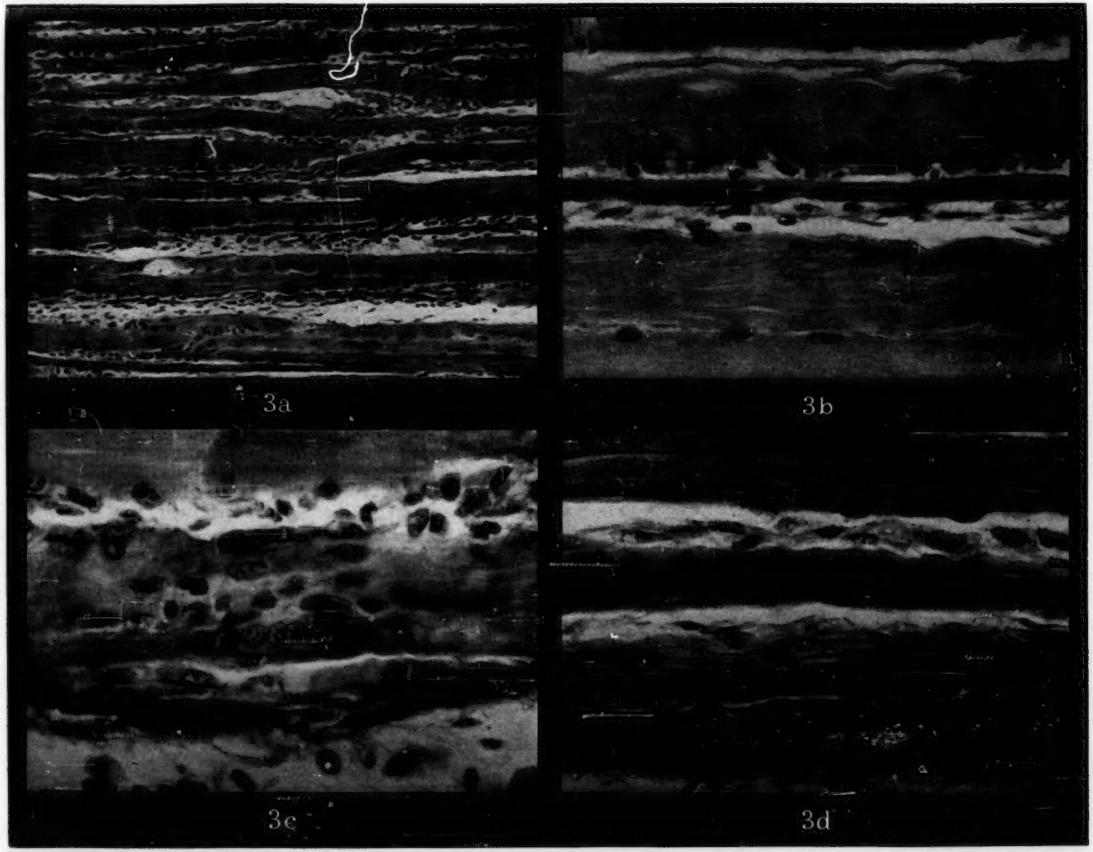

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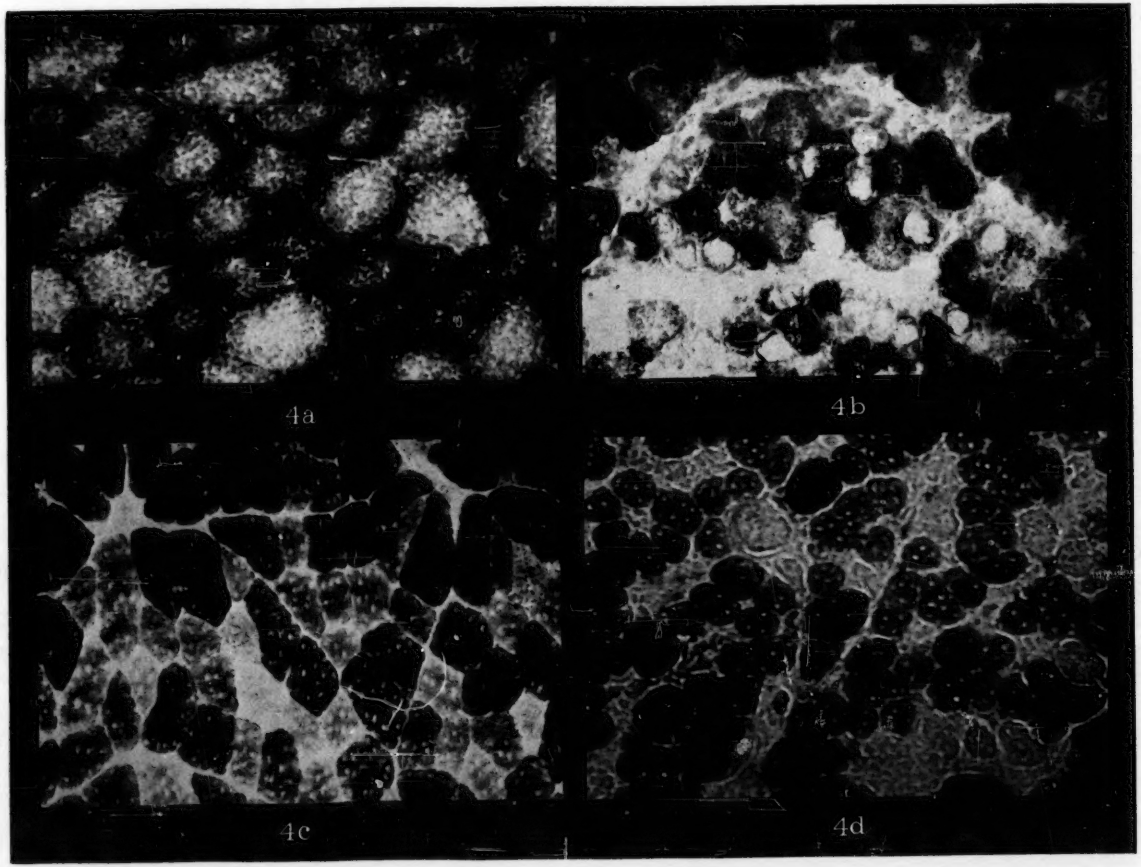

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tive enzymes studied, succinic dehydrogenase and DPN diaphorase showed almost no activity as expected, in the necrotic fibres (Fig. 4*a*, *b*). In the less injured fibres, the enzyme activity was relatively the same in intensity, but varied in distribution. In contrast with normal fibres in which the localization of the enzyme is subsarcolemmal, there was a migration towards the centre of affected fibres. Since both white and red muscle fibres were involved in this process, an apparent increase in enzyme activity resulted. Among other enzymes tested, the glucosan phosphorylase activity was found to be reduced in proportion to the damage in the fibres (Fig. 4*c*, *d*). On the other hand, leucine aminopeptidase, which is usually not demonstrable in normal fibres, was found to be present where histiocytic activity culminates.

DISCUSSION

Numerous factors must be considered when experimental lesions are compared with those occurring spontaneously either in animal or in man. The chronology and intensity of a pathological process often differ from one species to another. Tissue constituents and morphology are also determining factors in the genesis of lesions. The muscle lesions described here, although very acute in nature, are reminiscent of primary myopathies. For instance, the coagulation necrosis is a common feature of hereditary muscular dystrophy in the mouse (West and Murphy, 1960) and apart from a transitory oedema directly due to the action of PPD, the interstitial inflammatory reaction is secondary to the destruction of muscle fibre. Hence, no fibrotic reaction is demonstrable in this particular form of myodegeneration. With regard to the embryological type of regeneration of the muscle fibres and the nuclear rowing, this phenomenon seems non-specific, as it occurs in other types of myopathies. In many respects the present muscle lesions can be compared to those produced experimentally in the rat by plasmocid or in hamsters and guinea-pigs by vitamin E deficiency (Hicks, 1950 ; West and Mason, 1958; Goettsch and Pappenheimer, 1931); ; the latter type of lesions however develop much more progressively. There is also a close relationship between these degenerative lesions and the muscle changes found in mice suffering from hereditary muscular dystrophy, except for the presence of a fibrotic reaction. Modifications in enzyme morphology also provide valuable information on the genesis of these experimental muscle lesions. As far as the degree activity of both oxidative and hydrolytic enzymes is concerned, this change is indicative of the extent of damage inflicted on the muscle fibre. But the unusual distribution of these enzymes in less affected fibres seems more revealing. For instance, the fact that these enzymes disperse from the subsarcolemmal area towards the central part of the muscle fibre would indicate that the pathological process originates in the interstitial tissue. Such a dispersion phenomenon has already been pointed out in muscles of dystrophic patients (Dubowitz and Pearse, 1960) as well as in those of dystrophic mice. The phenomenon perhaps parallels central nuclear rowing. Recent studies performed in this laboratory also suggest that relatively slight alterations of the capillary permeability in the muscles is followed by enzyme migration in the fibres. In fact, it has already been established that paraphenylenediamine exerts its toxic action on the capillary wall of all the tissues studied to date (Geschickter *et al.*, 1958 ; Jasmin, 1961). It is only reasonable to suppose that the changes observed in the muscle

fibres are secondary to the capillary damage. In this connection, the studies of Beckett and Bourne (1958) concerning the determination of 5-nucleotidase in human dystrophic muscles would indicate that the pathological process primarily involves the interstitial tissue. Therefore, some aetiological similarities can be drawn between the PPD induced muscle lesions and those muscular disorders of primary origin.

SUMMARY

A histogenic study was made on the lesions occurring in the striated muscles of rats injected with dimethyl-paraphenylenediamine. These lesions develop within 24 hr., and consist in swelling and homogenization of muscle fibres. On the following days, the injured fibres undergo fragmentation and coagulation necrosis. The muscle segments act as foreign bodies and give rise to a histiocytic reaction with formation of multinucleated giant cells. Regenerative activity parallels the severity of damage to muscle fibres. It is characterized by nuclear rowing and neoformation of small basophilic fibres. In less than 20 days, the skeletal muscle restores *ad integrum* with no fibrosis or fatty tissue replacement. Lesions in the myocardium, which consist in granular degeneration and proliferation of mononucleated cells, become fibrotic. Modifications in the pattern of oxidative and hydrolytic enzyme distribution were also found.

We wish to thank Professor J. L. Riopelle for his kind suggestions in preparing this manuscript.

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RELATION OF LEUCOCYTE LIPID METABOLISM TO CELL AGE: STUDIES IN INFECTIVE LEUCOCYTOSIS*

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Received for publication June 29, 1961

LIPID synthesis from acetate in human whole blood *in vitro* occurs mainly in the leucocytes, some probably taking place in the platelets but none in the mature erythrocytes, although residual capacity for lipid synthesis persists in the reticulocytes (Marks, Gellhorn and Kidson, 1960; Pastore and Leonetti, 1958; Elsbach, 1959; Buchanan, 1960). Lipids synthesized by the leucocytes are transferred to the plasma and some at least are reutilized from the plasma by the leucocytes (Marks *et al.*, 1960; Lovelock, James and Rowe, 1960; Rowe, 1960).

Recent studies have shown high rates of lipid synthesis in myeloid leukaemia and low rates in lymphatic leukaemia (Kidson, 1961*a*; Kidson, Marks and Gellhorn, unpublished). These results raised the questions: Is the rate of lipid synthesis related to the age of leucocytes? Do the increased rates in myeloid leukaemia represent increased activity in young myeloid cells or do they indicate a more specific alteration in metabolism? Studies in myeloproliferative states (Kidson, 1961*b*) revealed low rates of lipid synthesis in polycythaemia vera, where large numbers of young cells of the myeloid series were present, suggesting that these young cells, in fact, had low rates of lipid synthesis, not high ones as might have been expected from the data on leukaemic cells. Increased incorporation of acetate into phospholipids relative to neutral lipids noted in leukaemia was not found in polycythaemia vera. These findings suggested that age was an unlikely explanation of the changes in myeloid leukaemia but the possibility of specific changes in metabolism rather than the presence of young normal cells in polycythaemia vera being responsible for lowered synthesis rates could not be excluded.

The present studies examine lipid synthesis in infective states and provide evidence of its relation to cell age.

MATERIALS AND METHODS

Subjects.—Studies were performed on the blood of 16 subjects in good health and of 12 subjects with infective leucocytosis. The subjects in good health used as controls had normal total and differential leucocyte counts. Those with infective leucocytosis had a variety of underlying pathological states and varying total and differential leucocyte counts as shown in Table I.

Incubation procedure.—Samples of 5 ml. venous blood containing 0·5 ml. of 2·5 per cent EDTA (ethylene-diamine-tetra-acetic acid disodium salt) in 0·9 per cent sodium chloride were placed in 25 ml. flasks with 0·5 ml. of 0·5 M glucose and 0·125 ml. of a mixture of sodium acetate-1-^{14}C with non-labelled sodium acetate (0·8 M sodium acetate containing 0·46 μc per ml.). The flasks were then gassed with 100 per cent oxygen, sealed, and incubated with shaking for 2·5 hr. at 37°.

* This work was carried out during the tenure of a grant from the Anti-Cancer Council of Victoria.

Separation of cells.—After incubation the cells and plasma were separated by dextran flotation using a modification of the method of Skoog and Beck as previously described (Marks *et al.*, 1960).

Lipid extraction and separation.—The lipids of leucocytes and plasma were extracted as described previously (Marks *et al.*, 1960) by the method of Folch, Lees and Stanely (1957), and separated into phospholipid and neutral lipid fractions by Eder's (personal communication) modification of the silicic acid column chromatographic method of Hirsch and Ahrens (1958).

Counting of radioactivity.—The radioactivity of the lipids was assayed in a liquid scintillation counter employing 0·3 per cent 2,5-diphenyloxazole and 0·3 per cent 1,4-bis-2-(5-phenyl-oxazolyl)-benzene in toluene as a solvent-phosphor system, with an absolute efficiency of 72 per cent and a background of 35 CPM. In all samples 10,000 counts were recorded and all counts were more than twice background. A full discussion of these methods has been published previously (Marks *et al.*, 1960).

RESULTS

Leucocyte morphology

From Table I it is evident that there was a considerable variation in total leucocyte count, but that in all cases neutrophils formed at least 70 per cent of this total. In most instances band forms constituted a high proportion of these. In 3 cases (1, 6 and 12) band neutrophils were less than 20 per cent of total leucocytes. No band neutrophils were present in the controls.

TABLE I.—*Pathological Data and Leucocyte Counts of Patients with Infective Leucocytosis*

No.	Pathology	Total WBC (Number $\times 10^6$/ml.)	Neutrophils (per cent)		Lympho-cytes (per cent)	Eosinophils basophils and monocytes (per cent)
			Lobed	Band		
1	Aortic stenosis—thoracotomy	24	61	9	24	6
2	Appendicitis—laparotomy	16	42	40	8	10
3	Abdominal aneurysm—laparotomy	14	48	42	10	—
4	Pelvic abscess	11	55	20	19	6
5	Subphrenic abscess	45	41	45	12	2
6	Lung abscess	49	60	10	17	13
7	Acute bronchitis	10	57	23	14	6
8	Pneumonia	25	35	53	8	4
9	Sub-acute bacterial endocarditis	10	62	21	11	6
10	Symbiotic gangrene	17	59	26	8	7
11	Wound infection	11	49	37	9	5
12	Septicaemia—pelvic abscess	40	64	13	16	7

TABLE II.—*Incorporation of Acetate-l-^{14}C into Total Lipids of Leucocytes and Plasma of Normal Subjects*

	Lipid syntheis ($\mu c. \times 10^{-3}/10^{10}$ cells)	Proportion in plasma (per cent)
Range of values (16 subjects)	23–50	57–80
Mean	34	73

Total lipid synthesis in controls

Values for incorporation of acetate-l-^{14}C into total lipids of leucocytes and plasma of 16 normal subjects are given in Table II. Of the mean total incorporation of 34 $\mu c \times 10^{-3}/10^{10}$ cells, 73 per cent appeared in the plasma fraction.

Total lipid synthesis in infective leucocytosis

In Table III are listed the rates of synthesis of total lipids in the 12 examples of infective leucocytosis. These gave a mean value of 13·5 $\mu c \times 10^{-3}/10^{10}$ cells. The variation observed in the proportion of labelled lipids appearing in the plasma probably represent variable plasma protein binding capacity for lipids, a phenomenon which has been discussed elsewhere (Kidson 1961*a*).

By comparison of Tables I and III it is evident that the three cases (1, 6 and 12) with the highest rates of lipid synthesis had relatively few band neutrophils, while those with the lowest rates of synthesis (2, 3, 5 and 8) had 40 per cent or more,

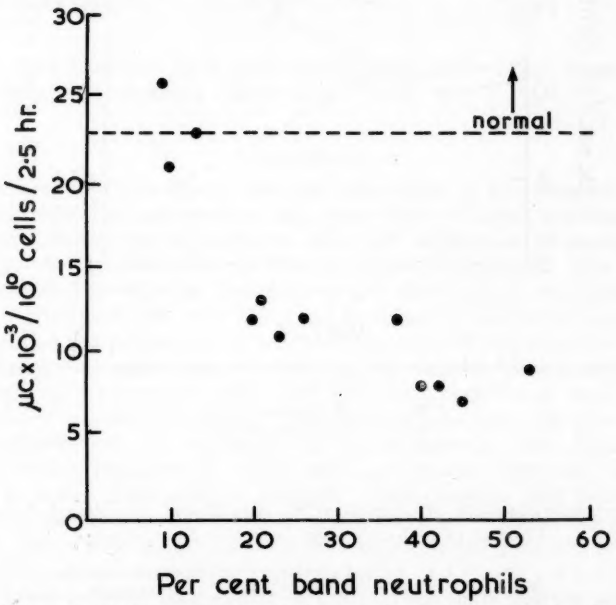

FIG. 1.—Relationship of band neutrophils to the rate of total lipid synthesis. The lower limit of the normal range is shown by the dotted lines.

of band neutrophils. This relationship is shown graphically in Fig. 1. A roughly inverse correspondence between the proportion of band neutrophils and the synthesis rate appeared to exist, but interpretation is somewhat complicated by the variations in numbers of cells other than neutrophils (lymphocytes, eosinophils, basophils, monocytes).

Changes during the course of infection

One patient with bronchitis observed over the course of 4 weeks to complete recovery showed the pattern illustrated in Fig. 2. There was a low rate of lipid synthesis with a leucocytosis (23 per cent band neutrophils) early in the illness, with a rise in the rate of synthesis corresponding to a fall in leucocyte count (to 0 per cent band neutrophils) with recovery.

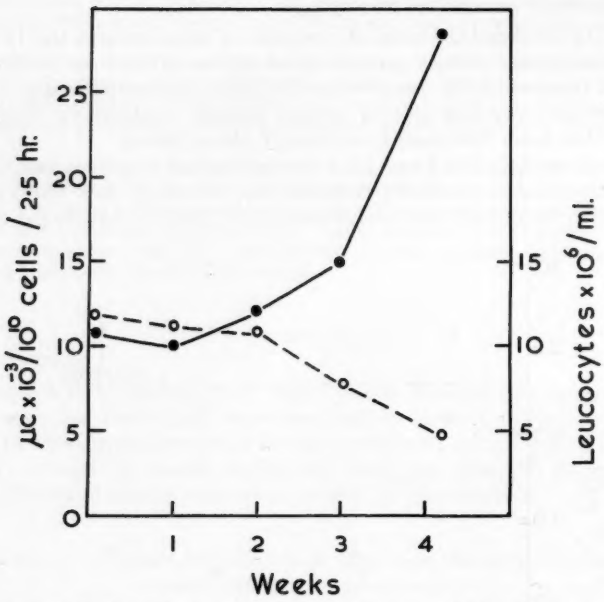

FIG. 2.—Changes in lipid synthesis rate and leucocyte count during the course of infection leading to recovery.

●———● Synthesis rate.
○- - -○ Leucocyte count.

TABLE III.—*Incorporation of Acetate-l-^{14}C into Total Lipids of Leucocytes and Plasma of Subjects with Infective Leucocytosis*

Subject	Lipid synthesis ($\mu c. \times 10^{-3}/10^{10}$ cells)	Proportion in plasma (per cent)
1	26	63
2	8	38
3	8	47
4	12	48
5	7	26
6	21	40
7	11	59
8	9	60
9	13	66
10	12	52
11	12	51
12	23	66

Fractional lipid synthesis

In Table IV are shown the figures for synthesis of phosolipids and neutral lipids in controls and infected subjects. There was possibly a slight proportional increase in synthesis of leucocyte phospholipid in the infective group, but this did not vary greatly from that of the controls. The differences between leucocyte

TABLE IV.—*Incorporation of Acetate-l-^{14}C into Neutral Lipids and Phospholipids*

	Leucocytes		Plasma	
	Neutral lipids (per cent)	Phospho-lipids (per cent)	Neutral lipids (per cent)	Phospho-lipids (per cent)
Normal—				
Range (16 subjects)	64–75	25–36	73–92	8–27
Mean	68	32	81	19
Infective—				
Range (12 subjects)	55–65	35–45	75–89	11–25
Mean	60	40	82	18

and plasma lipid fractions have been found consistently in all experiments and have been discussed previously (Marks *et al.*, 1960).

DISCUSSION

It is evident from this study that the leucocytes in the peripheral blood of patients with infective leucocytosis had lower rates of lipid synthesis than the leucocytes of normal control subjects, although differences of incorporation of radioactivity into lipid fractions, if present, were not marked. The lowest synthesis rates tended to occur in those cases with the highest proportions of band neutrophils. Synthesis rate rose to control levels as the leucocyte count returned to normal and as the percentage of band neutrophils fell towards zero.

It is generally accepted from morphological evidence that band neutrophils are young cells of the myeloid series, and that their presence in large numbers is an indication of a relatively young leucocyte population. It was also found in earlier experiments on leucocytes in polycythaemia vera (Kidson, 1961*b*) where considerable numbers of young cells are present, that the rate of lipid synthesis was lower than that in controls. The question was raised in those experiments as to whether this decrease was a function of the young normal cells or a specific pattern of metabolism of leucocytes in polycythaemia vera. The present evidence in infective leucocytosis suggests that young cells of the myeloid series have lower rates of lipid synthesis than do the more mature cells and that this pattern is the same as the one present in polycythaemia vera.

Elsbach (1959) reported that rabbit polymorphonuclear leucocytes showed increased lipid turnover during active phagocytosis *in vitro* and Sbarra and Karnovsky (1960) reported increase in the incorporation of acetate-l-^{14}C, glucose-U-^{14}C and ^{32}P into lipids of phagocytosing guinea-pig leucocytes *in vitro*, indicating increased lipid synthesis. The leucocytes in the present experiments, being taken from the peripheral blood, away from the actual infective site, could be considered as resting cells. If the results from rabbits and guinea-pigs are applicable to human subjects, it may well be that the low rates of lipid synthesis observed in the relatively young cell populations studied here in the resting phase would increase during active phagocytosis. This remains to be tested.

Previously it was reported that leucocytes in myeloid leukaemia have increased rates of lipid synthesis (Kidson, 1961*a*; Kidson *et al.*, unpublished) and again, the question of cell age was raised as a possible explanation with the suggestion that young myeloid cells in leukaemia might have a more active metabolism with

respect to lipids. The findings in polycythaemia vera and in the present study in infective leucocytosis of diminished lipid synthesis rates with increasing proportions of young myeloid cells rather than increased rates as might have been expected suggest that the increased rates observed in myeloid leukaemia represent alterations in metabolism of abnormal cells and are not a function of cell age. The additional finding of a disproportionate increase in synthesis of phospholipids relative to that of neutral lipids in myeloid leukaemia (Kidson, 1961*a*) which was not evident in polycythaemia vera or infective leucocytosis, supports this conclusion. Nothing is known, however, of the behaviour of myeloid cells younger than band neutrophils in comparison with the cells of acute myeloid leukamia.

With respect to neutrophils, then, it is evident that young cell populations have lower rates of lipid synthesis than do older cell populations. Such a conclusion is supported by the rough, inverse correlation established between the number of band neutrophils and synthesis rate, and by the rise in the rate of lipid synthesis during the course of an infection as the leucocyte pattern returns to normal. It is apparent, therefore, that lipid synthesis in neutrophil leucocytes is to some extent at least, age dependent.

SUMMARY

Lipid synthesis in human leucocytes was examined *in vitro* in blood from patients with infective leucocytosis and normal controls using acetate as substrate. The incorporation of acetate-1-^{14}C was measured in total lipids, neutral lipids and phospholipids of the leucocytes and plasma.

Low rates of total lipid synthesis were observed in infective leucocytosis, but the proportional synthesis of phospholipids and neutral lipids did not differ markedly from the controls. The lowest rates of synthesis occurred in the cases with the highest proportion of band neutrophils, and lipid synthesis rose to control levels as the total leucocyte count and the proportion of band neutrophils fell to normal.

The findings were considered to represent a dependency of lipid synthesis rate on cell age, with decreased synthesis in young cells of the myeloid series. They are discussed in relation to previous results in myeloid leukaemia and polycythaemia vera.

Gratitude is expressed to Dr. T. E. Lowe for helpful advice and suggestions in the preparation of the manuscript, and to the Department of Physiology, University of Melbourne, for permission to use the liquid scintillation counter for assaying radioactivity.

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HYALINE EMBOLI IN THE MICROCIRCULATION OF RABBITS DURING ANAPHYLAXIS

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Received for publication July 6, 1961

THE general manifestations of anaphylactic shock have been described many times. The phenomena vary from one species to another, but in each are somewhat constant. The unanaesthetized rabbit develops a marked pallor of the ears followed by irregularity of its breathing. The animal sways, collapses, and usually passes urine and faeces : its breathing becomes rapid, it loses consciousness, and finally after a few convulsive kicks, respiration ceases and death ensues. In the anaesthetized animal, death usually occurs quietly without convulsion. At autopsy, the characteristic finding is extreme dilatation of the right side of the heart with congestion of the liver and gastro-intestinal tract. Death is usually attributed to contraction of the branches of the pulmonary artery resulting in acute right heart failure. Generalized arterial constriction has been observed by arterioradiograph (Vallery-Radot, 1949).

Investigators who have studied the circulation in the living animal during anaphylaxis have noted not only changes in the calibre of the vessels, but also alterations in the flow of the blood. Abell and Schenck (1938), using a transparent chamber in rabbits' pinnae, observed white cells sticking not only to the vascular endothelium, but also to each other, forming clumps which blocked the circulation in many capillaries and venules. Ebert and Wissler (1951) noted aggregation of red cells, a change which may be described as sludging of the blood (Knisely, Elliot and Bloch, 1945 ; Knisely, 1951).

In addition to these changes, highly refractile masses, termed white or hyaline emboli, have been seen in the blood stream and have been observed to obstruct the flow of blood in the microcirculation (Burrage and Irwin, 1953). These emboli have been observed in the livers and lungs of rabbits, and in the spiral ligament of the guinea-pig's cochlea, not only in anaphylaxis but also in histamine poisoning (Irwin, Weille and Burrage, 1955).

The composition of these emboli is not known. They have been thought to be aggregates of platelets or white cells, antigen-antibody precipitates, fibrin or other protein precipitates (Irwin, Weille, York and Rappaport, 1959).

Although these hyaline emboli have been fully described in the living animal, little has appeared in the literature concerning their appearance in histological sections. Series of experiments have been undertaken to investigate their nature, and the present paper reports the histological appearances and staining properties of emboli in the lungs and other organs.

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METHODS

Sensitization of animals

Sixty-six albino rabbits were sensitized by injection of an emulsion of 1 ml. of Freund's adjuvant containing 50 mg. per ml. of bovine serum albumin (BSA), (Leskowitz and Waksman, 1960) 0·5 ml. being injected into each of two toepads. Approximately 1, 2 and 3 weeks later, booster intravenous injections of 10 mg. BSA in 1 ml. saline were administered. One to two weeks later, anaphylactic shock was induced by intravenous injection of 50 mg. of BSA with the animal in position for direct observation of the lung. This involved opening the chest under pentobarbital sodium anaesthesia and examining the lung using the quartz rod technique. Respiratory movements were abolished by continuous intratracheal insufflation of oxygen through a tracheotomy opening. The details of this method have been reported by Irwin and MacDonald (1953) and Irwin, Burrage, Aimar and Chesnut (1954).

The rabbits frequently died in anaphylactic shock within minutes, but when death did not appear imminent, the animals were killed within 10-30 min. of the shocking dose.

Preparation of sections

Immediately after death, the lungs were inflated with 10 per cent formol saline through the tracheotomy opening. The lungs were carefully removed from the chest and after adequate fixation, two blocks were taken and sections prepared by the usual paraffin wax technique. Great care was taken in cutting the lung to avoid undue mechanical distortion. In addition the heart (12 cases), kidney (9 cases), brain (6 cases), liver (9 cases) and spleen (3 cases) were also sectioned. Haematoxylin and eosin, PAS and Weigert's fibrin stain were used as routine. In selected cases the following stains were used: Mallory's phosphotungstic acid haematoxylin (PTAH) with sections previously mordanted with acidified alcoholic potassium dichromate for 30 min., Baker's acid haematin method, and picro Mallory stain (MacFarlane, 1944). Sections were examined for emboli and an estimate made of their number. They were classified as "few" when a considerable search was required to find them, and "many" when most low power fields (using a $\times 10$ objective and $\times 10$ eyepiece) contained emboli. The remaining sections were placed in an intermediate grade.

RESULTS

Emboli were found in the lungs of all animals and in the heart, liver, kidney and brain in all those cases in which these organs were sectioned. They appeared as eosinophilic hyaline masses and were found lodged in capillaries or impacted in arterioles where the diameter of these vessels narrowed. Thus they were present at the mouth of a branch of a vessel or at a point of bifurcation. Occasionally they were found lying free in a venule or arteriole. The site of occurrence and their appearance varied in different organs according to the different vascular patterns.

Appearance of emboli

Lung.—In 6 cases emboli were found lying free in large vessels producing clear areas in the midst of the red cells (Fig. 1). The structures did not appear to be attached to the wall of the vessel, nor was any evidence found to suggest that they originated as thrombi. Some emboli were surrounded by a rim of polymorphs which appeared to have stuck to their surface (Fig. 2). In arterioles emboli were frequently found at points of division (Fig. 3). The emboli themselves usually presented a homogeneous hyaline appearance although sometimes red or white cells were incorporated (Fig. 4). Occasional emboli were granular (Fig. 5). Elongated emboli were frequently found impacted in the capillaries where they distended the vessels and produced widening of the alveolar

septa. When the capillaries were seen in transverse section, the alveolar wall presented a characteristic beaded appearance (Fig. 6). Their distribution tended to be focal; one area associated with an arteriole showed nearly every capillary blocked whilst adjacent areas were relatively free.

Liver.—In the liver the emboli showed an even greater tendency to focal distribution than in the lung. Some lobules showed a large number of emboli in the sinusoids. Sometimes the hepatic veins contained emboli, but these did not appear to produce complete obstruction (Fig. 7).

Kidney.—In the kidney the greatest number of emboli were found in the capillaries of the glomerular tuft (Fig. 8). The kidney in fact was the organ other than the lung in which they were most easily found. A few were present in the capillary network around the tubules and occasionally a larger embolus was found in an afferent arteriole (Fig 9).

Emboli were found blocking the capillaries of the cortex of the brain and in the myocardium (Figs. 10 and 11). In the spleen they were scanty, being found blocking the Malpighian arterioles.

Frequency

Emboli were found in the greatest number in the lungs in all cases. No very close correlation was found between their frequency in other organs as compared with the lung. In particular, the number found in the heart muscle varied considerably from case to case and bore no constant relationship to the number found in the lungs. These findings may not, however, present a true picture since the focal distribution of emboli makes it difficult to get an accurate assessment of their relative numbers in various organs unless many sections are examined.

Staining reactions

The emboli stained pink with eosin: they retained the violet in Weigert's stain provided differentiation was not too prolonged. This latter stain was useful for identifying emboli when they were few in number and difficult to find in H and E sections. The PAS reaction was weakly positive in most cases with the emboli staining pink: a few, however, stained brighter pink. With PTAH the larger emboli stained deep blue, but their edges tended to be pink (Fig. 12). The small emboli showed some variation, but many were blue. All emboli stained deeply with picro-Mallory stain (Figs. 3, 6, 10 and 11). Baker's acid haematin method stained the emboli blue.

DISCUSSION

The composition of the emboli seen in anaphylactic shock is not known. They have been called white emboli and thought to be clumps of white cells (Abell and Schenck, 1938). Like most observers, we have seen white cells adhering to the endothelium of lung capillaries and in histological sections groups of polymorphonuclear leucocytes may be found. The emboli themselves, however, are not composed of white cells; they may contain them, but the cells are not an essential constituent and indeed are frequently absent. It is probable that the emboli present a surface to which polymorphs may adhere since some emboli are seen coated with these cells. It is evident that unless the basophilic nuclear staining

of the white cells is lost in some way, making their recognition difficult, the emboli are not composed of aggregates of these cells.

Weatherford (1935), studying the liver in anaphylaxis, observed the plugging of the central veins by hyaline masses. He regarded these as resulting from stasis of the blood caused by swelling of the parenchymal cells of the centre of the lobule. Individual red cells lost their identity and appeared to form a hyaline mass. The emboli which have been described in the living animal are colourless and quite unlike aggregates of red cells. The emboli in sections which we have observed may contain distinct red cells, thus making it highly unlikely that the whole mass is of compressed red cells (Figs. 4 and 12). There can be little doubt that neither the red nor white cells are essential constituents of the emboli.

The possibility of platelet emboli remains and indeed it is probable that emboli of different compositions occur. Some are hyaline (Figs. 4 and 12) and show no detectable structure, whilst others have a distinct granular appearance (Fig. 5). Antigen-antibody precipitates could also form embolic masses, but since emboli have been observed in histamine poisoning it is evident that they can be formed without antigen-antibody reaction.

The staining reactions of the emboli suggest that they contain fibrin. The deep staining with PTAH, the retention of methyl violet with Weigert's method and the positive acid picro-Mallory results all support this. Fibrin is generally stated to be PAS positive (Pearse, 1960), but the emboli themselves are not strongly stained by this method. The staining properties of fibrin formed *in vitro* have been shown to vary according to the manner in which it is produced Indeed it is probable that the protein " fibrin " gives none of the characteristic tinctorial reactions of ordinary " fibrin " as seen in histological sections (Gitlin and Craig, 1957). Neither the positive identification of fibrin nor its distinction from other similar substances like " fibrinoid " can therefore be satisfactorily demonstrated by ordinary histochemical methods. The emboli which we have demonstrated in anaphylaxis give characteristic reactions of fibrin, but the evidence that

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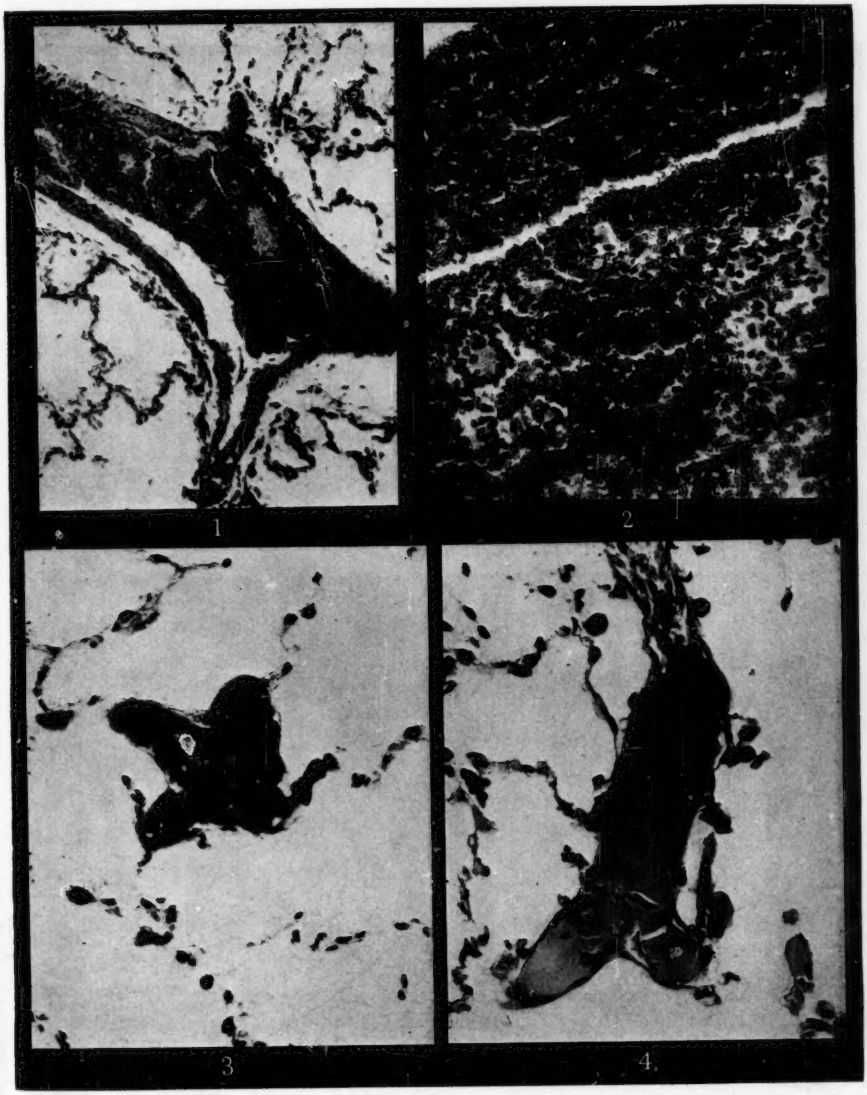
EXPLANATION OF PLATES

FIG. 1.—Emboli lying free in a small pulmonary artery. Haematoxylin and Eosin. ×215.
FIG. 2.—The contents of a small pulmonary vein showing emboli surrounded by a halo of polymorphonuclear leucocytes. Haematoxylin and eosin. ×395.
FIG. 3.—Embolus impacted at a point of trifurcation of a small pulmonary vessel. Picro Mallory stain. ×395.
FIG. 4.—Hyaline embolus impacted in a pulmonary vessel. The red cells contained in the embolus remain quite distinct. Haematoxylin and eosin. ×395.
FIG. 5.—Granular embolus in a pulmonary vessel. Several white cells are incorporated. Haematoxylin and eosin. ×733.
FIG. 6.—Emboli in small pulmonary capillaries producing a characteristic beaded appearance in the alveolar walls. Picro-Mallory stain. ×395.
FIG. 7.—Section of liver showing a hyaline embolus in a central vein. White cells, red cells and possibly platelets are also present. Haematoxylin and eosin. ×395.
FIG. 8.—Glomerulus showing obstruction of capillaries by several emboli. Haematoxylin and eosin. ×395.
FIG. 9.—Kidney glomerulus showing an embolus obstructing the afferent arteriole. Haematoxylin and eosin. ×450.
FIG. 10.—Embolus obstructing capillary in the cerebral cortex. Picro-Mallory stain. ×450.
FIG. 11.—Myocardial capillary showing an impacted embolus. Picro-Mallory stain. ×450.
FIG. 12.—Lung. Hyaline embolus showing a central deeply staining part (blue) with a pale (pink) edge. Incorporated red cells are black. Phosphotungstic acid haematoxylin. ×450.



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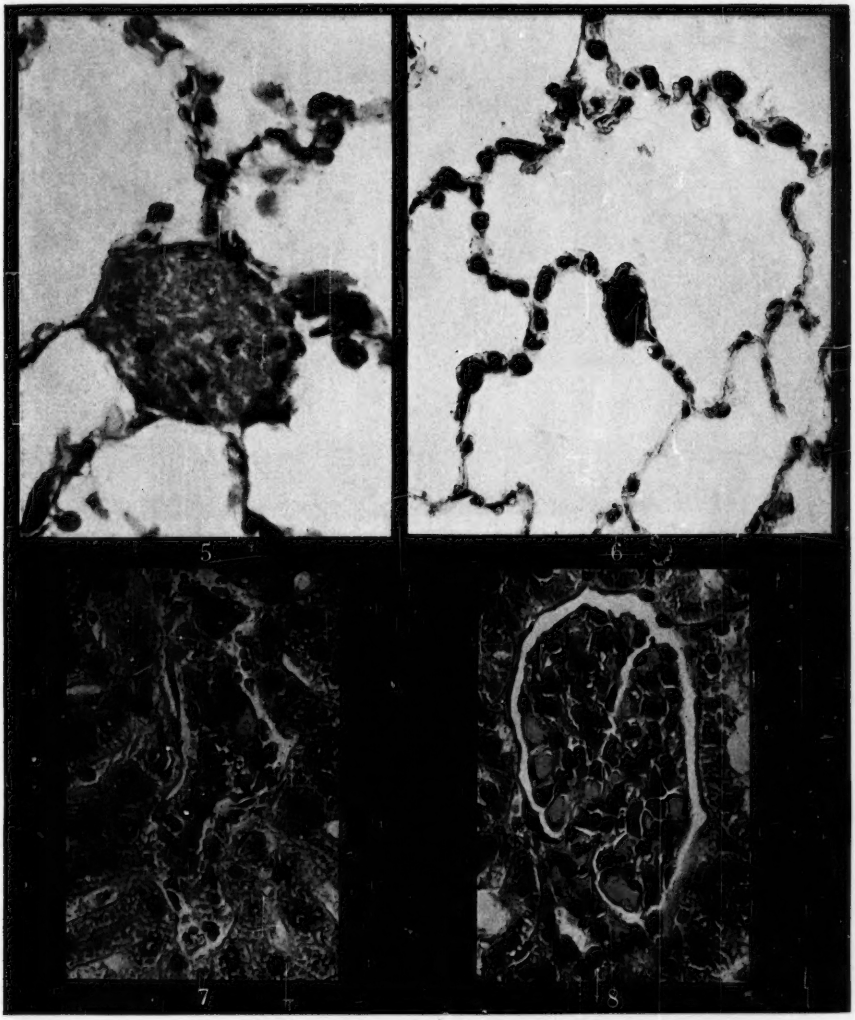

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9 10 11

12

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they are fibrin is not conclusive. Indeed it is probable that the emboli are not homogeneous since the PTAH method shows that certain parts of a single embolus stain differently from the rest.

The site of formation of emboli is not known. Their impaction in many different organs makes it unlikely that they are formed in any one particular organ. It seems probable that they are formed in the circulating blood stream throughout the body; chance incorporation of red or white cells favours this hypothesis.

The part played by emboli in the manifestations of anaphylaxis is difficult to assess. Obstruction of the pulmonary circulation is generally regarded as due to vascular spasm, but it is probable that mechanical blockage plays a part, for although the obstruction by the emboli is focal, blockage is nevertheless extensive. Whatever the part played by these emboli, it is evident that the changes in anaphylaxis are generalized. To state that death is due to constriction of the smooth muscle of the pulmonary vasculature is an obvious oversimplification of the problem (Burrage and Irwin, 1953). Many other organs are affected in anaphylaxis. Electrocardiographic irregularities have been reported and in the rabbit are so constant and early that they have been regarded as an independent occurrence and not secondary to other factors. Embolization in the heart may be an explanation of these cardiographic changes.

Although hyaline emboli have not been observed in the lungs of man during anaphylactic shock, it is probable that they occur. Their demonstration in cases of sudden death, suspected of being anaphylactic shock, might be of value in substantiating the diagnosis. Caution may, however, be necessary since similar embolic phenomena may occur in many conditions other than anaphylaxis.

SUMMARY

Anaphylactic shock was induced in 66 rabbits which had previously been sensitized to bovine serum albumin by 3 injections given at weekly intervals. Hyaline emboli were identified in sections of the lung in all animals. They obstructed arterioles and capillaries, producing in the latter a characteristic beaded appearance. The emboli were not composed of aggregated red or white blood cells, but gave many of the staining reactions generally regarded as characteristic of fibrin.

Emboli were also found impacted in small vessels in kidney, liver, heart, brain and spleen. It is suggested that obstruction by emboli plays a part in the production of some features of anaphylaxis. Their distribution favours the concept that they are formed in the circulating blood.

This work was performed whilst one of us (J. B. W.) was holding a British Postgraduate Travelling Fellowship. The work was in part supported by grants from the National Tuberculosis Association and Lakeside Laboratories Inc. We wish to thank Mr. A. L. E. Barron for the photographs, and Miss Irene M. York for the antibody estimations.

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THE RELATIONSHIP OF HYALINE EMBOLI TO OTHER MANIFESTATIONS OF ANAPHYLAXIS

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Received for publication July 6, 1961

In the preceding paper we described the presence of impacted emboli in the vessels of the lungs and other organs of animals subjected to anaphylactic shock (Walter, Frank and Irwin, 1961). In addition to the histological studies, the lungs of these animals were examined microscopically during the shock. This paper reports the correlation of these *in vivo* studies with the histological findings and the relationship of the emboli to the antibody content of the blood and the severity of the shock.

METHODS

Sensitization and antibody determination

Seventy albino rabbits were sensitized to bovine serum albumin (BSA) and shocked as previously described. Before anaesthesia, 20 ml. of blood were withdrawn for estimation of the circulating precipitin antibody. The antibody nitrogen content per ml. of serum was obtained by a quantitative method employing the Biuret reagent and the Beckman D.U. spectrophotometer (Kabat and Mayer, 1948). Taking the blood volume of the animal as 85 ml./kg. body weight, the result was expressed as total circulating antibody nitrogen.

In vivo *observations*

Lung.—Animals were anaesthetized with pentobarbitol sodium, tracheotomy performed and respiratory movements abolished by continuous intratracheal insufflation with oxygen (Meltzer and Auer, 1909). Thoracotomy was performed and the edge of the right middle lobe examined microscopically using the quartz rod technique: this method gave magnification of up to 900×. The equipment and method of study has been fully described by Irwin and MacDonald, 1953, and Irwin, Burrage, Aimar and Chesnut, 1954. When the lung was under observation, the shocking dose (50 mg. BSA) was injected and the reaction of the animals recorded. The time of appearance of emboli was noted and an estimate of their number made. This was recorded as few, moderate, or many. In some experiments 16 mm. cinematographs were taken.

Pinna.—Three animals had had ear chambers inserted previously and these were examined as well as the lungs during anaphylactic shock. The chambers used were constructed of perspex and mica and were a modification of the type used at Oxford (Sanders, Dodson and Florey, 1954).

Histological examination of the lungs

Sections of lung from 66 of the animals were examined for emboli and an estimate made of their number in arterioles and capillaries. Results were recorded for each type of vessel as follows:

\+ Very few emboli, requiring considerable search.
\+\+ Moderate number present.
\+\+\+ Large numbers seen; emboli present in most fields using ×10 objective and ×10 eyepiece.

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Although emboli in arterioles and capillaries were recorded separately, it was found that the numbers present in arterioles and capillaries were closely related. The maximum number of emboli possible was ++++++, and the minimum +. For some purposes of analysis the number of emboli was recorded as "few": 1 or 2+, "moderate": 3 or 4+ or "many" 5 or 6+.

RESULTS

Direct observation of the living animal during anaphylaxis

Of the 70 animals used for lung examination, successful observations were made in 60, and emboli seen in all cases except one. They usually appeared within 10 sec. of the shocking dose. Sometimes they were seen as early as 5 sec. but in one case they were not seen until 105 sec. after the injection. They varied from a few to great numbers. They were observed in arterioles, venules, and capillaries and as far as could be judged appeared simultaneously in all 3 types of vessels.

When large numbers of emboli were present in the observed field, the vessels usually became plugged and blood flow ceased in this area of the lung. Search in other fields, however, often revealed some patent vessels. The animal invariably died when blood flow had ceased in many of the vessels.

Two types of emboli were identifiable in the living lung: some appeared as rigid, clearly defined structures which readily became impacted, while others had poorly defined margins and changed shape during their passage through the vascular channels. Such emboli were able to pass through capillaries. Emboli were also seen in the 3 ear chambers.

Emboli in sections

Emboli were found in sections in all cases. The details of this study have been previously reported (Walter, Frank and Irwin, 1961).

Frequency of emboli in section and in vivo

There was some general correlation between the number of emboli seen in sections and *in vivo*. Thus of the 29 cases in which many emboli were observed in life, 17 revealed numerous emboli in section. Moreover, none of the 7 cases in which few emboli were seen in life showed many emboli histologically. The frequency of emboli in sections compared with the number seen in the same animal during life is shown in Table I.

TABLE I.—*Relationship between Number of Emboli Seen* In Vivo *and Number Seen in Section*

Number of emboli seen *in vivo*	Number of emboli seen in section			
	Few	Moderate	Many	Totals
Few	4	3	0	7
Moderate	5	4	4	13
Many	5	7	17	29
None	0	0	1	1
Totals	14	14	22	50

Relationship between number of emboli seen in section and antibody level

The figure shows that there is no relationship between the number of emboli seen in sections and the antibody content of the blood immediately before shock.

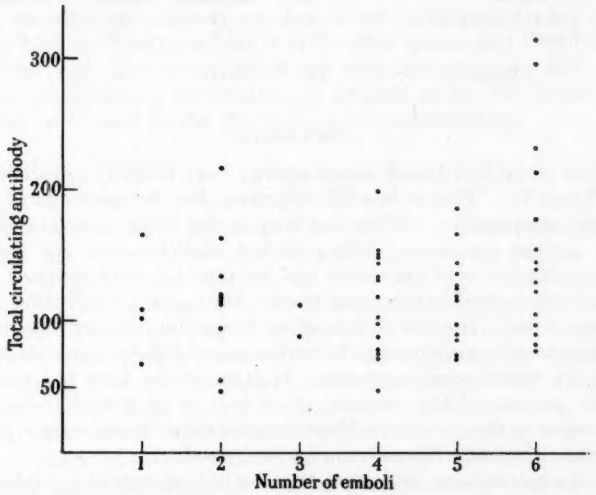

FIG.—Relationship between total antibody content and number of emboli seen in section. The antibody content of the circulating blood is expressed as mg. of nitrogen. The number of emboli is recorded in arbitrary units varying from few (+) to many (++++++). (See text.) Each point represents one animal.

Relationship between antibody level and severity of shock

All 5 animals with an antibody nitrogen content of over 200 mg. died while only 9 out of 25 with an antibody nitrogen content of less than 99 mg. died. The results are shown in Table II.

TABLE II.—*Relationship between Antibody Content and Fate of Animal*

Fate of animal	Circulating antibody nitrogen (mg.) 0–49	50–99	100–149	150–199	200–249	250–299	300+	Totals
Dead or dying	0	9	17	7	3	1	1	38
Lived	2	14	11	2	0	0	0	29
Totals	2	23	28	9	3	1	1	67

Relationship between number of emboli in section and severity of shock

Table III shows that the animals with the greatest number of emboli in section also show the highest mortality rate.

TABLE III.—*Relationship between Number of Emboli in Lung Section and Fate of Animal*

Fate of animal	Number of emboli seen in section			
	Few	Moderate	Many	Totals
Dead or dying . .	5	11	20	36
Lived . . .	11	6	9	26
Totals . .	16	17	29	62

DISCUSSION

The number of emboli found histologically only roughly paralleled those seen *in vivo* (see Table I). This is hardly surprising for the methods of examination are not strictly comparable. When the lung in the living animal is observed only a few vessels can be examined. Many emboli may be seen, but these may pass through the capillaries and therefore not be found in the sections of the lung. Furthermore, histological study has shown that the distribution of impacted emboli is often focal. Results obtained by observing one or two vessels *in vivo* would not, therefore, be expected to resemble exactly those obtained by examining many vessels by histological methods. It is probable that the sections gave a more accurate picture of the number of emboli present and these results were used in preference to the *in vivo* findings for purposes of comparison of numbers of emboli with the other manifestations of anaphylaxis.

The *in vivo* observations indicated that emboli appeared in the circulation in nearly all animals shocked. There was one exception in the present series, but previous work from this laboratory has shown that when animals die rapidly of anaphylactic shock, emboli do not always appear (Burrage and Irwin, 1953). Furthermore, the number of emboli found impacted in the lung is not closely related to the severity of the shock (Table III). Animals which showed the largest number of emboli histologically also had the highest death rate, but there are many exceptions to this generalization. The *in vivo* observations were similarly related to death, for whilst heavy embolization was usually associated with severe shock and death, some animals which died showed few emboli. It must be concluded that although the number of emboli present is related to the severity of shock, embolization is not the only factor concerned : other mechanisms must be involved and in some cases they are predominant. Nevertheless, the extensive plugging of vessels by emboli must play some part in the local or general manifestations of anaphylaxis.

Previous work has indicated that emboli contain fibrin with the cellular elements of the blood playing no important role in their formation (Walter, Frank and Irwin, 1961). They have, however, been regarded as antibody-antigen precipitates (Germuth and McKinnon, 1957), although the appearance of white emboli in histamine poisoning is against this view. It is possible that morphologically similar emboli could have different compositions, but comparison between the number of emboli seen and the antibody content of the blood (Fig.) shows that there is no relationship between the two. This would indicate that in anaphylaxis at any rate the emboli are not simple antigen-antibody precipitates.

The nature of the antibody concerned in the sensitization of animals to anaphylaxis has never been clearly defined. There is considerable evidence that the

degree of sensitization is related to the precipitin antibody content of the blood (Doerr and Russ, 1909). That these are not the only antibodies concerned has, however, been demonstrated by Kabat and his co-workers (1942, 1947 and 1949), who showed that non-precipitating antibodies were equally potent in sensitizing guinea-pigs for general anaphylaxis. Our finding (Table II) of the relationship between total circulating antibody content of the blood and the fate of the animal indicates that high antibody content is more often associated with death than is a low level. However, the correlation is not close and supports the view that the production of precipitating antibodies, as evidenced by the blood level, is not the only factor concerned in the development of sensitization.

SUMMARY

Anaphylaxis was induced in 70 sensitized rabbits. Direct examination of the lung using the quartz rod technique revealed hyaline emboli in 59 of the 60 animals successfully observed. In general, the number of emboli seen was related to the number found subsequently in histological sections of the lung. The focal distribution of emboli probably accounts for lack of exact correlation.

The number of emboli seen in sections of the lung bore no relationship to the precipitin antibody titre of the blood before shocking. This is put forward as evidence against their being simple antigen-antibody precipitates. The severity of the anaphylactic shock showed some positive correlation with the antibody titre and the number of emboli present histologically. The fact that this correlation is not close indicates that although the obstruction produced by the emboli may play a part in the symptomatology of anaphylaxis, this is not the only factor concerned.

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THE ROLE OF *BORDETELLA PERTUSSIS* AS AN ADJUVANT TO ANTIBODY PRODUCTION

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Received for publication June 13, 1961

RAMON AND ZOELLER (1926) first explored the effect of adding a bacterial vaccine to a toxoid, and the resultant serum antitoxin levels in man with TAB and diptheria toxoid were " at least as good as those obtained with the toxoid alone ". Unfortunately no toxoid concentration or diphtheria antitoxin titres were given nor were contemporaneous controls used. This reference and Ramon and Zoeller (1927) include the further statement that TAB vaccine was effective in increasing tetanus antitoxin levels. This effect of TAB vaccine was verified in horses by Ramon (1931) and in man by Maclean and Holt (1940).

Although Bordet (1936) was the first to mention the use of a mixture of diphtheria toxoid and *Bordetella pertussis* vaccine he gave no details. Greenberg and Fleming (1947, 1948) showed that the addition of either *B. pertussis* or TAB vaccine to diphtheria toxoid produced a 4-fold enhancement of antitoxin levels in guinea-pigs by Schick testing 19 days following subcutaneous injection. Fleming, Greenberg and Beith (1948) obtained results in children with *B. pertussis* and diphtheria toxoid similar to those found with guinea-pigs (1947). The enhancing action of *B. pertussis* on antibody production to diphtheria toxoid was confirmed by Ordman and Grasset (1948), Ungar (1952), Barnes and Holt (1955), and Barr, Fulthorpe and Llewellyn-Jones (1957) using guinea-pigs, by Faragó and Pusztai (1949) using rabbits and by Bousfield and Holt (1957) using children.

Although all these workers had found this adjuvant effect, Sauer and Tucker (1942) could not demonstrate any enhancing potency of *B. pertussis* on diphtheria toxoid in children. Also, Barr and Llewellyn-Jones (1953), using TAB vaccine with either two doses (0·5 Lf each) or one large dose (9 Lf) of tetanus toxoid in guinea-pigs, found that the response to tetanus toxoid was lower when TAB vaccine was administered simultaneously than when the toxoid was injected alone. They found an adjuvant effect on one occasion only, using a smaller dose of tetanus toxoid (0·2 Lf instead of 0·5 Lf).

In the current investigation the role of whole organisms and chemical fractions of *B. pertussis* and of *Escherichia coli* in increasing serum antitoxin levels to a standard antigenic stimulus of 5 Lf diphtheria toxoid was explored in the guinea-pig. The choice of antigen was based on the high degree of accuracy of the *in vivo* method of diphtheria antitoxin estimation.

MATERIALS AND METHODS

Diphtheria toxoid.—The strain of *Corynebacterium diphtheriae* used was G12/6, a variant of the classic American PW8. The method of culture, harvesting the toxin, toxoiding, puri-

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fication of the toxoid and final testing followed the procedure in WHO monograph (1953). The final concentrated, purified toxoid had a potency of 6300 Lf/ml. and a purity of 1940 Lf/mg. protein-nitrogen. The dosage level of 5 Lf was used throughout because for the strain of guinea-pigs employed this was the threshold dose—the smallest quantity which would stimulate all the animals in the group to produce a titratable level of antitoxin (>0·0002 units/ml.).

B. pertussis *vaccine.*—The strain PC3 was maintained in lyophilized form and was first subcultured on to a modified Bordet-Gengou diagnostic medium (WHO monograph, 1953) for vaccine production. The growth was washed off with liquid Cohen and Wheeler's medium after 48 hr. and incubated overnight at 37°. This suspension was then plated on solid Cohen and Wheeler's medium and incubated at 37° for 4 days. The organisms were removed with sterile saline, killed by heating to 60° for 1 hr., and preserved by the addition of 1/10,000 merthiolate. The concentration of vaccine was 6×10^{10} organisms/ml.

Escherichia coli *vaccine.*—The organisms, strain 2380, were grown on 2 per cent nutrient agar in sloped Thomson bottles. After incubation at 37° for 36 hr., and washing off with sterile saline the suspension, at a concentration of 8×10^{10} organisms/ml., was killed by heating at 100° for 10 min.

Preparation of purified lipopolysaccharide.—The method was based on Westphal, Lüderitz and Bister (1952). Equal volumes of 90 per cent phenol and suspension of killed organisms were heated to 68°, mixed and kept at that temperature for 30 min. with constant stirring. The mixture was cooled to 0° when it separated into 2 phases—an upper watery layer containing lipopolysaccharide and nucleic acid and a lower phenol layer containing mainly protein. Between these two phases was the bacterial residue. Separation of the phases was completed by centrifugation at 3000 rpm for 20 min. at 0° and the upper watery layer carefully withdrawn. Second and third extractions were made by adding a volume of water at 68° equal to that removed, and continuing as before. The three watery layers were combined and precipitated with 5 vol. of absolute ethanol in the presence of sodium acetate. The precipitate was recovered by centrifuging at 3000 rpm for 15 min., washed twice with absolute ethanol and resuspended in a minimum of distilled water to form an opalescent solution. This crude lipopolysaccharide solution, diluted to 250 μg./ml., was examined in a Beckmann ultra-violet spectrophotometer in the range 200–300 mμ., together with a control solution of yeast nucleic acid. Some absorption was shown by the extracted material in the region of 260–280 mμ, indicating contamination with nucleic acid. The degree of contamination was estimated by comparison with the observed standard absorption curve for yeast nucleic acid. Ultra-centrifugation at 78,000 ***g*** for 2 hr. (twice) resulted in the purified lipopolysaccharide being precipitated. This was redissolved in distilled water and, when examined in the spectrophotometer, showed no absorption at 260–280 mμ indicating no detectable amount of nucleic acid in the preparation.

The extracted lipopolysaccharide was tested as follows: (i) Production of hyperthermia. Rectal temperatures of rabbits were measured at regular intervals from 2 hr. before to 6 hr. following injection. A group of 3 rabbits (approx. 3 kg. each) was given 0·05 μg. lipopolysaccharide intravenously in 1 ml. of pyrogen-free saline. A control group received the saline only. (ii) Local Shwartzman effect. 20 μg. in 0·2 ml. was injected into the skin of a rabbit and 24 hr. later 20 μg. was given intravenously. After 18 hr. a haemorrhagic necrosis rapidly developed at the site of the first injection. Saline controls gave no reaction. (iii) Measurement of LD_{50} in mice. Mice (LAB grey) weighing 18–20 g. were used and results calculated using the method of Reed and Muench (1938). An LD_{50} of 70 μg. was estimated for *Esch. coli* lipopolysaccharide. That for the strain of *B. pertussis* used was 750 μg.–1 mg. (iv) Chemical analysis. Total nitrogen (1·5 per cent) and phosphorus (2·4 per cent) determinations were made on *Esch. coli* lipopolysaccharide and both values fell within the range estimated by Westphal and Lüderitz (1954).

Preparation of Lipid A.—Lipopolysaccharide was hydrolysed with N HCl for 30 min. at 100°, the precipitated lipid A washed with distilled water and taken up in chloroform while degraded polysaccharide was left in solution (Westphal and Lüderitz, 1954). Lipid A composed 12 per cent of the lipopolysaccharide in the strain of *Esch. coli* used and 19 per cent in *B. pertussis.*

Guinea-pigs.—The Porton strain of guinea-pigs was used throughout in groups of 12, or occasionally 9, animals in a group. These were albinos of both sexes weighing 300–500 g. Using a colour code the animals were individually identified and this enabled an accurate statistical analysis to be made of the serum antitoxin levels obtained.

Injection procedure.—All injections in the antibody response experiments were made subcutaneously into the ventral wall of the abdomen in a volume of 1 ml.

Storage of sera.—Guinea-pigs were bled by cardiac puncture 28 days after inoculation. Serum was always stored at 4° if it was to be used within 48 hr., otherwise stored at −20°.

Estimation of diphtheria antitoxin in serum.—Individual serum diphtheria antitoxin titres were estimated by a micro-method based on the technique of Romer and Sames (1909) and the geometric means were calculated for each group.

Mathematical analysis of results.—The "*t*-test" was used throughout to determine whether 2 sets of antitoxin titres, and their means were significantly different or could be regarded as similar responses.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S}\sqrt{\frac{n_1 \cdot n_2}{n_1 + n_2}} \quad \text{on } n_1 + n_2 - 2 \text{ d.f.}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means of the 2 groups, n_1 and n_2 are the numbers in each group, S is the square root of the pooled variance

$$S^2 = \frac{s(x_1 - \bar{x}_1)^2 + s(x_2 - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

RESULTS

Demonstration of adjuvant effect of B. pertussis *vaccine on antibody production to diphtheria toxoid*

Fig. 1 shows the mean antitoxin levels 28 days after inoculation when groups of guinea-pigs were given a constant amount of 5 Lf of toxoid and increasing con-

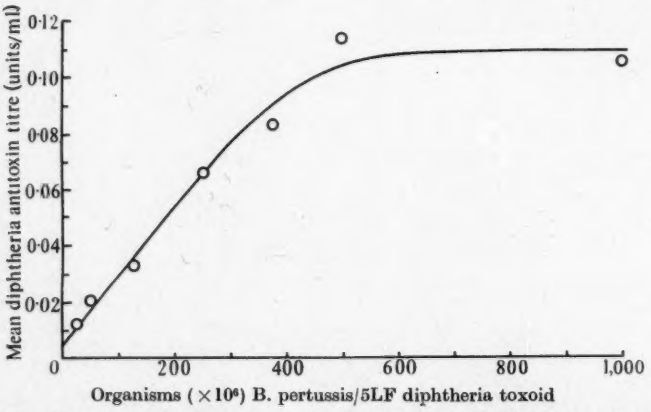

FIG. 1.—Relation between antitoxin response and dose of *B. pertussis* vaccine administered with 5 Lf toxoid, 28 days of inoculation.

centrations of *B. pertussis* vaccine from $2{\cdot}5 \times 10^6$ to 1×10^9 organisms. The antitoxin response increased with the concentration of vaccine.

This result agrees with that found in extensive preliminary tests showing that the adjuvant effect is maximal between $5–10 \times 10^8$ organisms per 5 Lf diphtheria toxoid. Above this ratio excess organisms did not further enhance antibody production. Neither was it decreased, *i.e.* the response with 4×10^{10} organisms per 5 Lf was not significantly different from that with 1×10^9, $t = 0{\cdot}24$ on 22 d.f. $p > 0{\cdot}1$.

The maximum degree of adjuvance shown in this experiment was 42-fold.

Preliminary tests to determine the fraction of B. pertussis *vaccine responsible for the adjuvant effect*

Supernatant fluid, washed bacteria, heated vaccine (100° for 60 min.), a preparation of cell walls and complete *B. pertussis* vaccine were tested at the optimal level of 1×10^9 organisms or its equivalent.

All these fractions showed appreciable increases in antitoxin titres over the controls with toxoid alone and were not significantly different in their response from the complete vaccine.

Since the complete vaccine and these various fractions were all subsequently found to contain lipopolysaccharide, it seemed probable that this component was responsible for its adjuvant action. It was therefore decided to extract pure lipopolysaccharide from *B. pertussis* organisms by the phenol/water method.

Ability of fractions from phenol/water extraction of B. pertussis *organisms, including lipopolysaccharide, to enhance antibody production*

When *B. pertussis* organisms were exhaustively extracted using the phenol/water method of Westphal and Lüderitz it was found that lipopolysaccharide composed approximately 5 per cent of the microbe in the strain used (Table I). By calculation 1×10^9 organisms, the optimal dose of vaccine, contains 1·73 μg. lipopolysaccharide.

TABLE I.—*Quantitative Extraction of Lipopolysaccharide from* B. pertussis *Organisms by Phenol/Water Method*

	Wt. (g.)	Wt. recovery (per cent)
Starting material (200 ml. vaccine at 1·1 $\times 10^{11}$ organisms/ml.)	0·770	—
Insoluble bacterial residue	0·309	40·1
Phenol layer (protein)	0·236	30·6
Water layer (crude lipopolysaccharide)	0·181	23·5
Lipopolysaccharide	0·0381 } 0·179	4·95
Nucleic acid	0·1409 }	
Total recovery	—	94·3

All fractions obtained in the extraction were tested at 1×10^9 organisms or its equivalent with 5 Lf diphtheria toxoid. The responses from the phenol layer and nucleic acid were not significantly different from the control whereas lipopolysaccharide significantly enhanced the production of diphtheria antitoxin at all the levels tested. Of this material 0·2 μg. showed a 6-fold and 500 μg. a 30-fold increase in antibody levels compared with toxoid alone. Fig. 2 shows that, as with the complete vaccine, an optimal value was reached, in this case between 0·5 and 5·0 μg., above which additional lipopolysaccharide did not further increase adjuvance. Since the calculated equivalent lipopolysaccharide for the optimal level of vaccine, 1×10^9 organisms, was 1·7 μg., it would appear from these experiments that the lipopolysaccharide alone was responsible for the enhancement of antibody associated with *B. pertussis* vaccine. Similar results were also obtained with lipopolysaccharide from *Esch. coli*. From Table I 0·2 μg. lipopolysaccharide would be equivalent to $1{\cdot}18 \times 10^8$ organisms and 0·5 μg. to $2{\cdot}95 \times 10^8$ organisms. In the dose/response experiment with complete vaccine these levels gave a geometric mean of 0·035 and 0·075 units antitoxin per ml. respectively

(Fig. 1), which compare well with 0·025 and 0·060 units per ml. found in this experiment. From these conclusions it would not be expected to find any enhancement of antibody production associated with other fractions from the phenol/water extraction. As stated before, this was so except in the case of the bacterial residue which gave a just significant adjuvant effect. Having been extracted seven times, it was not thought that any lipopolysaccharide remained in this residue since after the third extraction no precipitate was formed when alcohol was added to the watery layer. However, this residue produced in rabbits the typical hyperthermic reaction associated with lipopolysaccaride and it was therefore assumed that a minute quantity of this material remained in spite of repeated extraction.

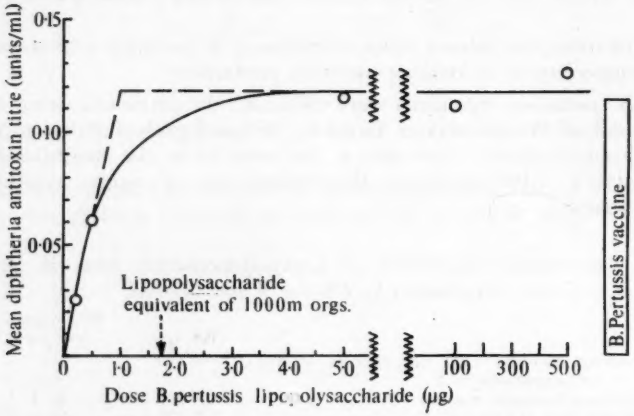

FIG. 2.—Response of guinea-pigs at 28 days to 5 Lf diphtheria toxoid and various doses of *B. pertussis* lipopolysaccharide.

Chemical fraction of lipopolysaccharide responsible for adjuvance

Lipid A was precipitated and degraded polysaccharide left in solution when lipopolysaccharide was hydrolysed with N HCl. Table II shows the adjuvant effect of *Esch. coli* lipid A upon diphtheria antitoxin production in that 10 μg. gave a 9-fold and 150 μg. a 10-fold adjuvance. Lipopolysaccharide demonstrated the usual enhancement but degraded polysaccharide when administered in an amount equivalent to that found in the parent lipopolysaccharide (88 μg.) produced no adjuvant effect. Although lipid A appeared to be the fraction responsible for the activity of *Esch. coli* lipopolysaccharide, the response was less than that achieved by the complete material. This difference is just significant at the highest level tested ($0{\cdot}02 < P < 0{\cdot}05$, $t = 2{\cdot}23$ on 16 d.f.). Even when the results from 50 μg. and 150 μg. were combined this difference was still significant when compared with the lipopolysaccharide ($0{\cdot}01 < P < 0{\cdot}02$, $t = 2{\cdot}52$ on 25 d.f.) and from Table II it can be seen that 150 μg. was well above the optimum level of lipid required. *B. pertussis* lipid A (19 per cent of the lipopolysaccharide) similarly was the active moiety. Here again the response was less than that

TABLE II.—*Effect of* E. coli *and* B. pertussis *Lipopolysaccharide (LP) and Components on Diphtheria Antitoxin Production*

Group / Results	5 Lf toxoid alone	5 Lf toxoid +100 μg. *Esch. coli* L.P.	5 Lf toxoid +10 μg. *Esch. coli* lipid A	5 Lf toxoid +50 μg. *Esch. coli* lipid A	5 Lf toxoid +150 μg. *Esch. coli* lipid A	5 Lf toxoid +88 μg. *Esch. coli* degraded poly-saccharide	5 Lf toxoid +10 μg. *B. pertussis* L.P.	5 Lf toxoid +50 μg. *B. pertussis* lipid A
Serum antitoxin titre. Group geometric mean (units/ml.)	0·003	0·108	0·030	0·040	0·038	0·004	0·133	0·036
log σ	0·61	0·37	0·38	0·46	0·47	0·53	0·50	0·61
p (cf. 5 Lf toxoid alone)	—	$<0·001$	$0·001<p<0·01$	$<0·001$	$<0·001$	$>0·2$	$<0·001$	$0·001<p<0·01$

achieved by the complete lipopolysaccharide ($0{\cdot}01 < P < 0{\cdot}02$, $t = 3{\cdot}96$ on 16 d.f.) even when the excess of lipid was administered.

DISCUSSION

In demonstrating the enhancing action of *B. pertussis* vaccine on the potency of diphtheria toxoid the results agree with the findings of the majority of workers. The failure of Sauer and Tucker (1942) to show this effect may be due to a number of reasons. They gave young children 3 subcutaneous injections at 3-weekly intervals of diphtheria toxoid and *B. pertussis* vaccine either mixed before injection or injected at the same time in the same arm, but at different sites, and Schick tested them 6 weeks after the last injection. Experiments designed to demonstrate the mode of action of these adjuvants (Farthing, unpublished) showed that with high Lf doses of toxoid no enhancement of antitoxin levels resulted, and when toxoid and vaccine were injected at different sites but at the same time some adjuvant action did occur. Farthing (1960) found that when the responses of guinea-pigs receiving diphtheria toxoid with *B. pertussis* vaccine were compared with those receiving toxoid alone there was no significant difference after 3 injections whereas there had been a 50-fold enhancement after the first injection. These observations, together with the possibility that the Schick test was not adequate in their experiment to show differences in antibody levels when compared with direct serum antitoxin measurement, may explain their apparent failure to demonstrate the adjuvant activity of *B. pertussis* on diphtheria toxoid.

Greenberg and Fleming (1947) showed that as the number of *B. pertussis* organisms was increased up to the highest level used in the experiment—$1{\cdot}6 \times 10^{11}$ with 40 Lf of diphtheria toxoid, the immunizing efficiency of the toxoid was also increased. Barnes and Holt (1955) found that with 3·5 Lf diphtheria toxoid and 1×10^9 *B. pertussis* organisms the degree of enhancement was 4·4-fold, although when the amount of vaccine was increased to 2×10^9 the adjuvance was only 1·2-fold. They concluded that there was an optimum ratio of *B. pertussis* to diphtheria toxoid above which the adjuvant effect was decreased. The experiments reported here showed that there was a peak of adjuvance between 5–10 $\times 10^8$ organisms per 5 Lf toxoid but above this ratio excess organisms did not further increase, nor indeed decrease antibody production.

The lipopolysaccharide prepared from *B. pertussis* and *Esch. coli* clearly demonstrated an adjuvant effect upon antibody production. A number of workers had found that various endotoxin preparations produced an increase in antibody production. Stuart and Windel (1951) using pyromen, a preparation from *Pseudomonas sp.*, found that when this was administered to rabbits with crystalline hen's egg albumin, precipitin titres to the albumin appeared earlier and were at least 50 per cent higher than in the controls. Nicholas and Bubel (1952) found that rabbits treated simultaneously with *Salmonella typhi* endotoxin and *Pasteurella tularense* vaccine showed increased antibody production over animals which received vaccine only. Condie, Zak and Good (1955) observed enhancement of antibody production in rabbits against crystalline bovine serum albumin by simultaneous injection of meningococcal endotoxin. An optimum dosage of 1/100–1/500 dilution of the endotoxin was found and the administration of larger amounts only resulted in lesser adjuvance. Johnson, Gaines and Landy

(1956) using *Salm. typhi* endotoxin in a number of experiments on rabbits found that 5 μg. was sufficient to produce a 20-fold increase in antibody levels when 2 mg. crystalline ovalbumin was used as antigen. This adjuvance was shown by both intravenous and subcutaneous routes of inoculation.

Johnson *et al.* (1956), using typhoid lipopolysaccharide failed to augment antibody levels in the guinea-pig to 5 Lf of diphtheria toxoid employing both the intracardial and subcutaneous routes of inoculation. Doses of lipopolysaccharide from 10–200 μg. and a secondary injection of the toxoid and endotoxin did not raise the antibody titres above those controls which received diphtheria toxoid only. Rabbits, as opposed to guinea-pigs, gave a 30-fold enhancement of antibody level when they administered 3 intravenous injections of 20 Lf toxoid and 5 μg. endotoxin. Although the material was isolated from a different species of gram negative organism, it is difficult to explain why these results differ. The importance of this will be discussed elsewhere (Farthing, unpublished).

In this work the lipopolysaccharide fraction of the *B. pertussis* organisms has been found to be the active part. Johnson *et al.* (1956), found that when complete typhoid vaccine was given under similar experimental conditions to the lipopolysaccharide it did not increase antibody levels above crystalline ovalbumin only. A monovalent vaccine of *Salm. typhi* and a triple vaccine containing *Salm. paratyphi A*, *Salm. schottmülleri* together with *Salm. typhi* were both tested and gave no effect, although the former contained approximately 3 μg. and the latter 10 μg. of lipopolysaccharide. Pittmann (1957) stated that the adjuvance shown by *B. pertussis* vaccine is greater than would be expected from the adjuvant effect of the endotoxin fraction of the organism alone but no data or evidence were given. The experimental results described here do not support this statement.

Ikawa, Koepfli, Mudd and Niemann (1953) and Westphal and Lüderitz (1954) have concluded that in the endotoxin complexes the antigenically specific polysaccharide and protein are linked by lipid A, the polysaccharide being bound by D-glucosamine and the protein by amino-dicarboxylic acids:

Phosphorylated polysaccharide
|
(D-glucosamine)——————acid labile
|
Lipid A
|
(Amino-dicarboxylic acid)——split by phenol/water
|
Protein

The lipid A fraction was observed to be the component responsible for the antibody enhancement of diphtheria toxoid. This was seen to be neither toxic (with *Esch. coli* up to at least 750 μg. in mice compared with LD_{50} of 70 μg. for parent lipopolysaccharide) nor pyrogenic (with *Esch. coli* at least 5 μg. whereas 0·05 μg. of parent material gave more than 2° F. rise in temperature). Though the lipid A of the lipopolysaccharide of both *B. pertussis* and *Esch. coli* were the only active fraction, even in excess they did not produce the same degree of adjuvance and this was significantly different from the parent material. Possibly molecular size and the degree of dispersion are of importance in adjuvant activity.

SUMMARY

Mean serum antitoxin levels measured 1 month after the inoculation of groups of guinea-pigs with heat-killed *Bordetella pertussis* vaccine in addition to 5 Lf diphtheria toxoid were 40-fold higher than in controls which received toxoid without vaccine. Maximal effect was given by 5–10 $\times$ 10^8 organisms and the response remained at the same level for higher doses of organisms. Lipopolysaccharide and lipid A effectively replaced whole heat-killed bacterial cells in the same test system although antitoxin levels were somewhat lower with the lipid A extract.

The author is indebted to Professor A. Neuberger and Dr. L. B. Holt for helpful discussion, Mrs. P. Pearce for technical assistance and Mr. T. Norris for his care of the animals.

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WATER CONTENT OF THE BRAIN IN ANOXIC-ISCHAEMIC ENCEPHALOPATHY IN ADULT RATS

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Received for publication July 12, 1961

IT has long been appreciated that the cerebral effects of anoxia may not be functionally apparent until several hours afterwards (Richardson, Chambers and Heywood, 1959). If the brain is examined microscopically the first changes in the nerve cell cytoplasm are seen after 18 hr., but neurone loss with a glial reaction are not seen until 24 hr. after the initial anoxic episode (Meyer, 1960). By contrast, following acute ischaemia of a portion of the brain, degeneration of neurones commences at 6 hr. (Denny-Brown and Meyer, 1957).

It is of considerable interest that death of neurones following anoxia is delayed for such a long period considering how metabolically active this cell is known to be, suggesting that anoxia is not the sole agent responsible for cell death.

An experimental system has been devised by Levine (1960) to produce brain damage in rats by a combination of ischaemic and anoxic anoxia. The purpose of this is to overcome the notorious resistance of the brain of this animal to anoxia. Many of Levine's experimental animals showed some swelling of the brain accompanying the nerve cell loss, and in the following work an attempt is made to investigate the role of changes in water content of the brain following anoxic injury.

MATERIALS AND METHODS

Animals.—Adult white Wistar rats of either sex and weighing 180–220 g. were employed.

Anoxic ischaemic insult.—The right common carotid artery was ligated in the neck under ether anaesthesia, 6–12 hr. later hypoxia was induced by placing the animal in a horizontal gas jar, 40 cm. in length and 7·5 cm. in diameter, into which nitrogen flowed at such a rate as to render the animal cyanosed and comatose with a respiratory rate of 62–66/min. (Levine, 1960).

If apnoea occurred the animal was immediately removed from the nitrogen atmosphere, artificial respiration performed and as soon as spontaneous breathing was re-established, returned to the gas jar. Atmospheric oxygen was then flushed out of the cylinder by increasing the flow of nitrogen to 6 l/min. for 10 sec.

The gas was saturated with water by bubbling through a Wolff bottle, the water in which was maintained at 24°. A thermometer was fixed to the inside of the cylinder and the temperature was controlled by the use of a 100 W electric light bulb outside the cylinder.

Water content of the brain.—The animals were killed by lightly anaesthetizing with ether and then rapidly opening the thorax and severing the inferior vena cava. The calvarium was rapidly opened with bone forceps and the brain removed, the forebrain being separated from the hindbrain by cutting through the brain stem at the level of the superior colliculi. The forebrain and hindbrain were divided into right and left halves.

Each portion of the brain was weighed (to the nearest 0·1 mg.) on a dry watchglass before and after attaining constant weight in a drying oven (115°).

Before weighing, the heated watchglass was cooled over anhydrous calcium chloride in a dessicator.

Exchange of plasma protein in the brain.—This was measured by injecting ^{131}I-labelled homologous serum intravenously 30 min. before killing the animal. The serum was prepared by the method of Veall, Pearson and Hanley (1955).

At exsanguination, 0·02 ml. of blood was pipetted into 2 ml. of isotonic saline and the radioactivity of this and portions of the brain were measured in a well-type scintillation counter.

After the radioactivity of the brain was measured the latter was weighed and the dry weight estimated.

Calculation of results.—These were expressed as a blood equivalent (the number of ml. of blood which contains the same radioactivity as 1 g. dry weight of brain).

Example

Dry weight estimations showed :

R. forebrain contains 80·5 per cent water, *i.e.* 4·12 ml. water/g. dry weight.
L. forebrain contains 77·0 per cent water *i.e.* 3·35 ml. water/g. dry weight.

Thus the right forebrain contains an excess of 0·77 ml. water/g. dry weight.

Radioactive protein studies showed :

The blood equivalent of the right forebrain is 0·14 ml./g. dry weight.
The blood equivalent of the left forebrain is 0·076 ml./g. dry weight.

Thus the right forebrain has a differential blood equivalent of 0·064 ml./g. dry weight.

From this it is assumed that every 0·77 ml. increment of water in the right forebrain contains 0·064 ml. blood equivalent of activity. But the radioactive isotope is attached to plasma protein, therefore, every 0·77 ml. increase in water content in the right forebrain contains the same amount of protein as in 0·064 ml. blood, therefore every ml. increase in water content of the right forebrain has a protein content 0·064/0·77 that of the blood (*i.e.*, 8·3 per cent of the blood protein value).

Palliation of oedema.—A group of animals which had been exposed to a similar anoxic ischaemic insult were given 3-hourly intravenous injections of 30 per cent urea (2 g. urea (kg. bodyweight)). As in the untreated group, the animals were killed at varying intervals after the anoxic episode and water content of the brain was estimated.

Assessment of brain damage.—In some of the animals a single 1·5 mm. coronal slice was removed from the caudal part of the forebrain prior to drying. This portion of brain was examined histologically.

RESULTS

Environmental temperature

At 21°, 23·5° and 25° no lesions were seen after 1, 24, 48, 72 hr. and 6 weeks following ligation of the right common carotid artery.

The rats remained functionally unaffected during periods of observation of up to 6 weeks and histological examination of the brain revealed no abnormality.

Nitrogen exposure

Following ligation of the right common carotid artery, exposure to nitrogen for 25, 30, 50 and 60 min. at 21° produced no brain lesion in animals assessed up to 6 days later, but at 23·5° a 25 min. exposure resulted in lesions in 2 out of 6 animals. Thirty min. of hypoxia at this temperature gave histological changes in 5/8 rats. The cerebellum and brain stem showed no lesion but the forebrain was affected on the right side. Contrary to Levine's findings (1960), the median portions of the forebrain were not affected and no damage was seen in the left side of the brain.

The sequence of events appeared to be as follows :

Phase of swelling.—This was visible about 6 hr. after the injury and persisted for up to at least 6 days in some cases. There was no change in the cells at the

beginning of this period, but the affected regions took on a spongy appearance and the midline structures were obviously displaced away from the side of the lesion.

Degeneration of neurones.—Degenerative changes were seen after 18 hr. in neurones in the right half of the forebrain. The areas affected were the cerebral cortex (all layers)—except in its paramedian portions, the lateral group of thalamic nuclei, globus pallidus, putamen, claustrum, olfactory lobes and hippocampus. The medial thalamic nuclei, the cingular region of the cerebral cortex and nuclei around the third ventricle were spared.

Proliferation of glia.—This was seen after 24 hr. as an increase in the number of microglial cells, and was followed by activation and proliferation of astroglia.

A prolongation in the time of exposure in nitrogen to 60 min., or raising temperature of the cylinder from 23·5° to 25° during administration of the gas produced oedema which was macroscopically visible within 2–3 hr.

Anoxia

After a 30 min. period of anoxia at 23·5°, the water content of the right forebrain rose above that of the contralateral side at 4 hr., but showed no significant increase before this time (Table I). A maximal difference was attained at 19–25 hr.

TABLE I.—*Water Content of the Forebrain after a 30-min. Period of Anoxia (ml./g. dry weight)*

Interval after anoxia (hr.)	Difference in water content of right–left halves of forebrain: Mean	S.E. of mean	
0–3	−0·09 ($n = 3$)	0·05	
4–18	0·44 ($n = 5$)	0·24	$t = 2·92$, $P = 0·05$
19–25	1·05 ($n = 4$)	0·53	
26–30	0·41 ($n = 2$)	0·23	$t = 7·1$, $P < 0·001$
31–73	0·27 ($n = 11$)	0·04	

In animals which exhibited no gross cerebral damage after 30 hr. no significant excess of fluid was measured in the right forebrain at this time, but if severe destruction of nervous tissue was seen the ipsilateral forebrain contained an appreciable excess of water 30–73 hr. afterwards (Fig. 1).

The hindbrain was unaffected and the water content of the right and left sides was equal in all cases. Table II shows that up to 8 hr. after a period of anoxia, the water accumulation increases with the length of the period of hypoxia. However, after 25 min. of hypoxia no appreciable oedema was observed.

Radioactivity

Table III shows a similar increase in radioactivity per unit volume of excess water accumulation in the right forebrain at 20, 24 and 72 hr.—at which times oedema is present to a marked degree and the cerebral vasodilation, which follows

anoxia, is not expected to have persisted (Kety and Schmidt, 1948). Thus in these animals the oedema fluid in the right hemisphere contained about 1/20 of the protein of blood.

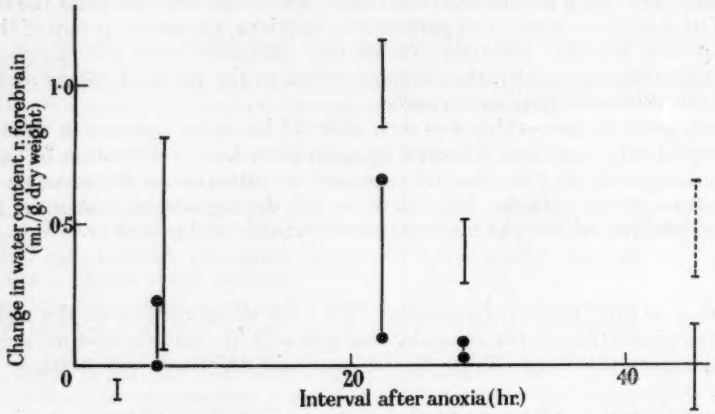


FIG. 1.—Effect of parenteral urea on water content of right forebrain in an anoxic ischaemic encephalopathy.

Urea treated animals ●——●
Untreated animals with no gross brain damage ⊢——⊣
Untreated animals with gross brain damage ⊢- - - -⊣

TABLE II.—*Change in Water Content of Right Forebrain after Varying Periods of Anoxia*

Period of anoxia (min.)	Interval after anoxia (hr.)	Change in water content of right forebrain (compared with left) (ml./g. dry wt.)
25	8	0·04
30	1	−0·12
30	4	0·10
50	2	0·77
60	4	1·19
0	Interval after ligation 6	−0·14
0	Interval after ligation 24	−0·04

EXPLANATION OF PLATES

FIG. 2.—Transverse section of rat brain through optic chiasma. Ligation of right common carotid artery (right side of brain on right side of photograph), 18 hr. after anoxia. Showing displacement of mid-line structures to the left, swelling of right side of brain, loss of nerve cells in the right hippocampus. ×7. Cresyl fast violet.

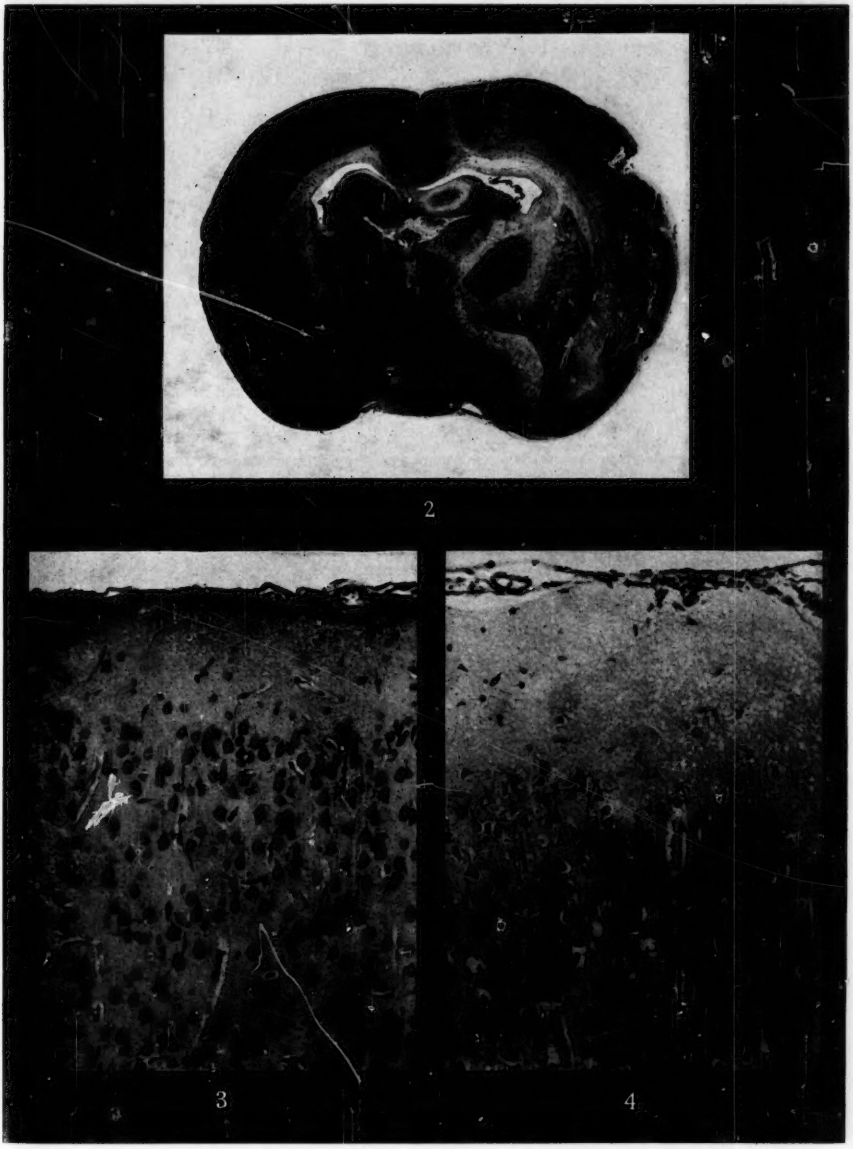
FIG. 3.—Left temporal cortex in Fig. 2. Normal structure. ×166. Cresyl fast violet.

FIG. 4.—Right temporal cortex in Fig. 2. Swelling in all layers. Degeneration of majority of neurones. ×166. Cresyl fast violet.

FIG. 5.—Similar to Fig. 2 six days after anoxia. Persistence of swelling on the right side, necrosis of the right cerebral cortex. ×7. Cresyl fast violet.

FIG. 6.—Left hippocampus in Fig. 5. Normal structure. ×166. Cresyl fast violet.

FIG. 7.—Right hippocampus in Fig. 5. Degeneration and loss of neurones with glial reaction. ×166. Cresyl fast violet.



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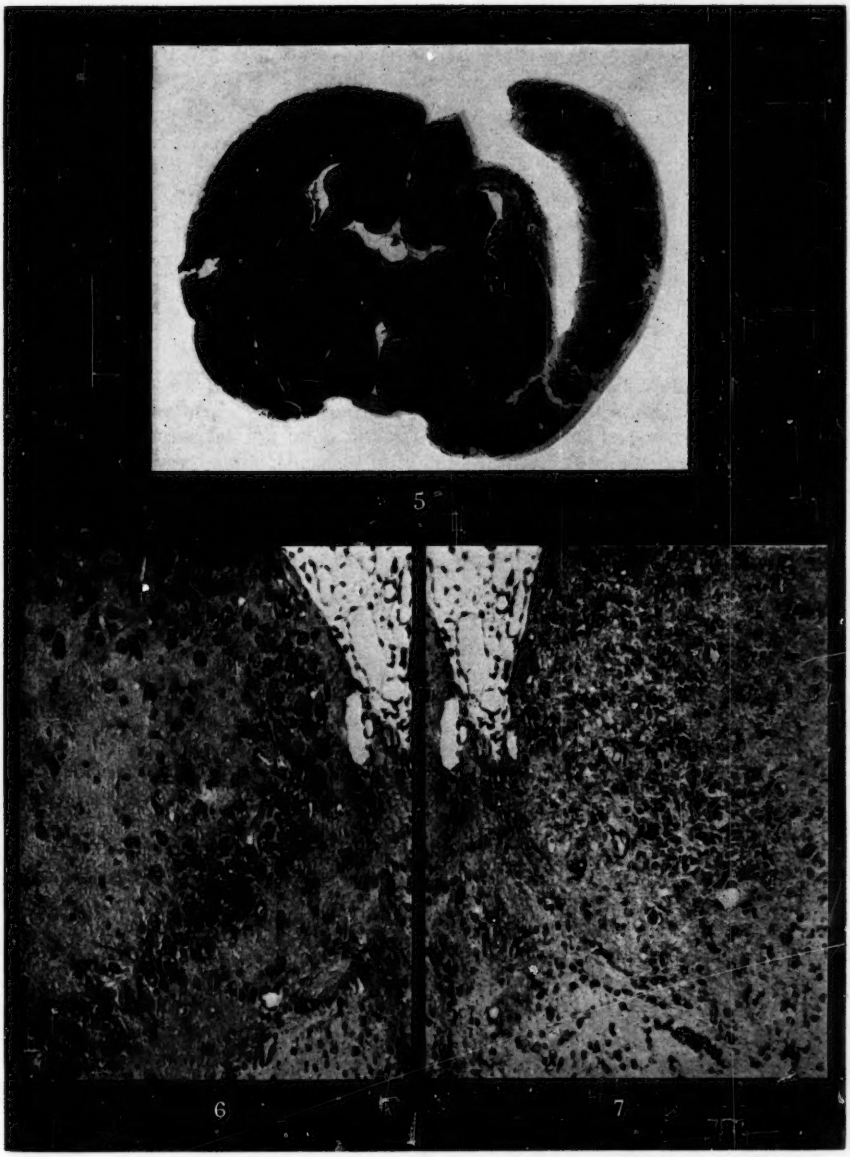

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TABLE III.—*^{131}I-Labelled Protein Space in the Forebrain following a 30-min. Period of Anoxia (expressed as ml. of Blood Containing the Same Amount of Radioactivity/g. dry weight of brain)*

Interval after anoxia (hr.)	Difference in water content of right–left halves of forebrain (ml./g. dry wt.) (A)	^{131}I labelled protein space			$\frac{B}{A}$
		Left forebrain	Right forebrain	Difference right–left forebrain (B)	
4	0·10	0·110	0·071	0·039	0·39
8	0·58	0·312	0·118	0·194	0·34
18	0·64	0·126	0·057	0·069	0·11
20	1·03	0·142	0·084	0·058	0·06
25	1·17	0·120	0·069	0·051	0·04
73	0·67	0·116	0·085	0·031	0·05

In the first 18 hr. after anoxia considerable vasodilation was observed by naked eye in the brain—most marked on the right side—and this would account for the high concentration of radioactive protein found in the right forebrain at this time. Nevertheless, there is only one-quarter of protein concentration at 18 hr. compared with that at 4 hr., indicating some decrease in the cerebral vasodilation between 4–18 hr.

Functional effects

The animals showed little disturbance on recovering consciousness after being removed from the atmosphere of nitrogen until 6–18 hr. afterwards. At this time there could often be detected diminution of muscle tone and power in the left hind limb and poverty of spontaneous movement in the left forelimb. Some such animals, if severely affected would tend to walk in a circular anticlockwise path. Less than one-sixth of the animals exhibited fits and all of these had an increased water content of the affected portion of the brain of more than 1 ml./g. dry weight. When fits occurred they started at 15–20 hr. and preliminary results showed that such animals always died within 6 hr. of the first fit.

Over half of all animals showing gross brain damage after 30 hr. were hemiparetic up to the end of the experiment.

Animals which were subsequently found to have no gross cerebral lesion were usually hemiparetic up to about 25–30 hr., but subsequently improved. Three-quarters of the hemiparetic animals showed a rise in water content of the right forebrain of 0·5–1 ml./g. dry weight. Some animals, killed before 10 hr., were hemiparetic but showed only a slight increase in cerebral water content.

Urea

The effect of urea administration on cerebral oedema in the 3 groups of animals to which it was administered is shown in Fig. 1 and Table IV.

At 19–25 hr. the difference between treated and control animals was significant ($t = 2{\cdot}3$, $P < 0{\cdot}05$), whilst during the preceding period the difference was not significant ($t = 1{\cdot}3$, $P = 0{\cdot}2$).

Although there was an impression that the functional state of the treated animals was superior to that of the untreated ones, it was difficult to express this in a quantitative manner which would permit statistical assessment. Therefore,

TABLE IV.—*Effect of Intravenous 30 per cent Urea on the Difference in Water Content of the Right and Left Halves of the Forebrain in Anoxic Ischaemic Encephalopathy*

Interval after 30-min. period of anoxia (hr.)	Drug administered	Difference in water content of right–left halves of forebrain (ml./g. dry wt.) Mean	S.E. of mean	
4–18	Nil	0·44 ($n = 5$)	0·24	$t = 1·3$
	Urea	0·13 ($n = 4$)	0·08	$P = 0·2$
19–25	Nil	1·05 ($n = 4$)	0·53	$t = 2·3$
	Urea	0·34 ($n = 4$)	0·20	$P = < 0·05$
26–30	Nil	0·41 ($n = 2$)	0·23	n too small for t test
	Urea	0·11 ($n = 2$)	0·01	

another set of experiments was performed in which the period of hypoxia was lengthened from 30 to 45 min. (Table V).

TABLE V.—*Effect of Parenteral Urea on 48-hr. Survival following a 45-min. Period of Anoxia after Ligation of the Right Common Carotid Artery*

	Died	Survived
Urea treated animals	2	15
Control animals	8	6

$\chi^2 = 7·2$
$P < 0·01$

Under these circumstances whereas 57·1 per cent of untreated animals died within 48 hr., only 11·8 per cent of the treated animals succumbed. The difference between these two groups was statistically significant ($\chi^2 = 7·2$, $P < 0·01$).

DISCUSSION

Oedema of the brain in both man and animals following anoxia has been observed by several workers (Hoff, Grenell and Fulton, 1945 ; Lucas and Strangeways, 1952 ; Earl, Baldwin and Penfield, 1953 ; Lindenberg, 1955 ; Richardson, Chambers and Heywood, 1959). Lindenberg suggests that this is the mechanism by which anoxia causes neuronal degeneration in man. He postulates that in some areas of the brain oedema produces a rise in tissue pressure which compresses small vessels and results in damage to cells which may thus be primarily ischaemic in nature.

The current work presents some experimental confirmation of Lindenberg's hypothesis in showing a rough correlation between rise in water content of the brain and functional disturbance to the animal. This does not prove that the cerebral oedema is harmful—as it may merely accompany a functional ab-

normality and play no part in its pathogenesis. The urea experiments show, however, that reduction in cerebral oedema by the use of this drug not only lowers the mortality rate from anoxic-ischaemic brain damage, but probably also reduces the severity of hemiplegia in survivors. Thus cerebral oedema, in these experiments, appears to play a part in the disturbance of brain function which follows anoxia.

The results also indicate that cerebral oedema is not the sole factor interfering with normal activity of the brain under these circumstances; hemiplegia was observed to be present in some animals in the presence of only a small degree of fluid accumulation. It is, therefore, possible that in this work anoxia can damage the central nervous system directly, and further injury can result from the post-anoxic cerebral oedema, the two effects being additive.

The work of Richardson *et al.* (1959), indicates that oedema of the brain may immediately follow an anoxic episode. In the current investigation there was a delay of about 4 hr. before a rise in cerebral water content occurred, but it was observed that the more severe the hypoxic episode, the earlier the oedema first appeared. ^{131}I-labelled protein in these circumstances could be either intravascular or extravascular. Thus differences in the radioactivity of the two cerebral hemispheres may be due to changes in either of these. Twenty-four hr. after the hypoxic episode cerebral vasodilation is not likely to be present; also, the estimated protein content of the accumulated fluid in the affected hemisphere was 1/20 that of the blood. From this evidence it is suggested that this fluid is extravascular (*i.e.* oedema) and contains only a small amount of protein.

Crocket (1956) has investigated the protein content of different varieties of oedema fluid and has shown that in states in which there is an excessive accumulation of fluid which is poor in protein, the capillaries are intact and the lymphatic clearance is normal, but that there is some abnormality of Starling's cycle. It seems unlikely that the capillary hydrostatic pressure had not returned to normal 24 hr. after hypoxia, also there is no evidence that the intravascular or extravascular colloids acutely decrease in this condition. Electron microscopy studies (Torack, Terry and Zimmerman, 1959) suggest that the extravascular intercellular space in the central nervous system is either very small in volume or does not exist at all and that cerebral oedema fluid accumulates in the cytoplasm of the glial cells, the stimulus for which may be, in the current work, an increase in electrolyte concentration in this cell.

The mechanism by which anoxia can cause cerebral damage following ligation of one common carotid artery is not known. One possibility is that even though blood continues to circulate in both halves of the circle of Willis following unilateral common carotid artery ligation, the ipsilateral vessels contain blood at reduced pressure and the increased rate of blood flow during an anoxic episode is submaximal. In this manner an important protective mechanism is impaired in the ipsilateral cerebral hemisphere, and when the animal is placed in nitrogen the oxygen availability to this portion of the brain is even more severely reduced than to other parts.

It also might be postulated that immediately after the anoxic episode there is a functional disturbance in the brain which could be independent of damage to the nerve cell by oedema, but the two effects may be additive—and together produce cell death. As oedema is delayed in onset, so the death of the neurone is similarly delayed.

Reduction of oedema would be expected to prevent neuronal destruction in those cases in which the initial anoxic stress was not sufficient to do this outright, or to minimise it when a dual mechanism (anoxia and oedema) is responsible for it. A lowering of the body temperature during anoxia by reducing the metabolic demands of the brain without proportionally slowing the cerebral circulation (Kleinerman and Hopkins, 1955) would similarly protect the nerve cells from damage, but if applied after the anoxic episode it could only minimise that portion of the damage which can be attributed to the secondary oedema.

SUMMARY

Anoxic-ischaemic encephalopathy in the rat is associated with cerebral oedema.

The oedema does not commence until after 4 hr. and reaches a maximum at 19–25 hr.

Some protection against this type of brain injury is produced by intravenous 30 per cent urea (2 g./kg. 3-hourly) after the anoxia or by lowering the environmental temperature to 21° during the anoxic episode.

The cerebral oedema fluid contains a low concentration of protein, and thus must form in the presence of intact capillaries. It is suggested that its accumulation may be due to a rise in electrolyte concentration in neuroglia cytoplasm.

I am grateful to Professor G. Payling Wright and Dr. J. B. Cavanagh for the use of their laboratories and also for their constant advice and encouragement. I would also like to thank Professor P. E. Polani for helpful discussions about the experiments and the manuscript. Mr. R. Passingham was kind enough to undertake the photographic work.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BRUCELLA ABORTUS*

I. ISOLATION OF *BR. ABORTUS* FROM BOVINE FOETAL TISSUE

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Received for publication July 21, 1961

THIS paper and others to follow in the series will describe attempts to identify chemically some of the mechanisms by which *Brucella abortus* causes contagious abortion in cattle and to see how far any important finding applies to other species of *Brucella*. In cattle, brucellosis is a disease of the sexually mature animal; young animals are relatively resistant. There are two phases of the disease, a slow invasion of the adult tissue producing the chronic, relatively mild disease which is also found in non-ungulates, and the dramatic overwhelming infection of the placenta and foetus in pregnant animals. Three aspects of bovine brucellosis were chosen for study. These are discussed briefly below but the literature on brucellosis is not reviewed since this has been done elsewhere (Larson and Soule, 1950; Stableforth, 1959).

Brucellosis is an intracellular disease and the ability of virulent strains of *Br. abortus* to survive and multiply within phagocytic and epithelioid cells appears to be a major factor in the invasiveness of this relatively feeble pathogen for adult tissue. It follows, that it is important to learn of the mechanism by which *Br. abortus* resists the cellular environment which is so destructive of other pathogens (*e.g. Bacillus anthracis*, *Pasteurella pestis*) and attempts will be made to identify the chemical basis of this special form of host parasite relationship.

In brucellosis of pregnant cows, the predilection of *Br. abortus* for foetal rather than adult tissue is well known (Stableforth, 1959). General pathological findings indicate an especially heavy infection in the foetal cotyledons, the chorion and the purulent foetal fluids (Smith, 1919; Payne, 1959). The cause of the predilection is a major unknown factor in the pathology of contagious abortion since once a heavy growth of the pathogen has occurred in these foetal tissues the well known endotoxin of *Br. abortus* could cause the characteristic abortion (Huddleson, 1953; Rudler and Thomas, 1960). Although the lack of immunity mechanisms probably has some effect, this cannot be the main reason for foetal infection, otherwise most pathogens would attack foetal tissue in the same way as *Br. abortus* and this is not so. A more specific basis for the preferential growth of the pathogen seems likely and attempts will be made to identify this.

Finally we are interested in the chemical basis of immunity to brucellosis (for review of immunogenic preparations see Stableforth, 1959). The best vaccines are live avirulent strains of *Br. abortus*; non-living vaccines are ineffective unless

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injected with adjuvant when some protection is afforded, which is however less than that produced by live preparations. Extracts of *Br. abortus* have been described which immunize guinea-pigs and mice and precipitate with various antisera. The degree of purity of these preparations is vague as is their relevance to immunity in cattle. Attempts will be made to obtain more precise information on the nature of the immunogenic product of *Br. abortus* and whether it is, in any way connected with the chemical basis for intracellular growth. Any findings might then be extended to vaccination against infections with *Br. melitensis* (where at present a live avirulent vaccine is more effective than any non-living preparation) and *Br. suis* (where at present there is no effective live or dead vaccine).

Our approach to some of these problems has been that which proved successful in similar studies of *B. anthracis* and *Past. pestis* (Smith, 1958 ; 1960). The fluids and tissues of an infected host and extracts of the organisms isolated from them were examined for compounds responsible for the pathological effects of the infection. These compounds were then purified either directly or after they had been demonstrated in cultures, *in vitro*.

This paper describes a quantitative survey for *Br. abortus* in the tissues of pregnant cows when they were about to abort 2–3 weeks after experimental infection with virulent *Br. abortus*. The survey fully confirmed the existence of a heavy infection in the cotyledon tissue, the chorion and the purulent foetal fluids. It underlined the importance of an investigation of the peculiar localization of the organisms and indicated the source of sufficient *Br. abortus* grown *in vivo* for chemical examination. The remainder of the paper describes the separation of *Br. abortus* from the infected bovine tissues. In this work *Br. abortus* had to be freed from their intracellular habitat and we were guided more by the work of Segal and Bloch (1956), who separated intracellular *Mycobacterium tuberculosis* from infected mouse lungs, than by our previous work on the separation of the extracellular *B. anthracis* and *Past. pestis* from infected body fluids.

METHODS

Infection of pregnant cows and collection of infected tissues

Through the kindness of the Director of the Agriculture Research Council's Field Station at Compton, Berks, pregnant cows were obtained which had been entirely free from contact with *Br. abortus* infection. When 5–6 months pregnant, the cows were infected with *Br. abortus* (strain 544, 1×10^{12}, intravenously) ; the organisms were grown for 48 hr. on tryptic meat agar, suspended in 10 per cent broth-saline and stored at 0° for 3 days until a viable count was known (on tryptic meat agar containing 5 per cent Fildes digest peptic sheep blood). The animal was observed hourly from the 17th day after infection until abortion was imminent (3–4 weeks). It was then promptly killed and the intact uterus removed and placed on a tray which was designed to enable all the foetal fluids to be drained off and collected. The uterus was dissected and the necrotic foetal cotyledons were separated from the maternal cotyledons. All the following foetal tissues were collected: cotyledons, allantoic and amniotic fluid (usually mixed), chorion, and foetal oedema fluid (found in thoracic and peritoneal cavities). The entire maternal cotyledon tissue was also removed but only weighed aliquots were taken of the rest of the relevant foetal and adult tissues. If the tissues were not examined immediately, they were stored at —20°.

Estimation of numbers of Br. abortus *in infected tissues*

The number of organisms in each tissue was estimated from counts of stained films of a known aliquot of the tissue which had been macerated in an overhead macerator (Townsend, Mercer and Co.). The films were prepared in a standard manner as follows. One loopful

of a suitably diluted macerate was spread on a slide over a circular area of 1 cm. diameter. The film was dried and stained by a modification of the Ziehl Neelson method (10 per cent carbol fuchsin 3 min., wash ; 0·5 per cent acetic acid 5 sec. ; wash 2 min. ; 2 per cent methylene blue 30 sec.). The method was standardized against Helber chamber counts of a culture of *Br. abortus*. Using a ×6 eyepiece and a ×95 oil immersion objective, and counting at least 50 fields from at least two separate films of the material, an average count of 30 organisms per field was $\equiv 1 \times 10^9$ ($\pm$ 50 per cent) organisms per ml.

Separation of Br. abortus *from infected foetal cotyledon tissue and purulent foetal fluids*

Br. abortus was separated from foetal cotyledon tissue (0·5–1 kg.), the purulent amniotic (0·5–1 l.) and allantoic (3–4 l.) fluids, and the calf oedema fluid (200–500 ml.).

The fluids were centrifuged for 40 min. at 18,000 rpm in the batch Rotor of the Spinco Ultracentrifuge at 0–3°. The supernatants were filtered through Millipore, and kept at −20° until they were examined for the extracellular or easily removed surface products of *Br. abortus*. *Br. abortus* was obtained from the centrifugal deposit (100–300 ml.) by a process summarized in the Fig.

Infected foetal cotyledon tissue was macerated 4 times for 1 min. in an overhead macerator (Townsend, Mercer and Co.) in a safety cabinet. The macerate was centrifuged at 0–3° for 30 min. at 2000 rpm in the International Model PR1 centrifuge. A cotyledon extract (200–300 ml.) was removed and treated as described for the foetal fluids (see above). *Br. abortus* was obtained from the centrifugal deposit (CM ; 300–700 ml.) by a process summarized in the Fig.

The separation summarized in the Fig. resulted in the final preparation of *Br. abortus* grown *in vivo* and the sucrose wash (SW) which was filtered through Millipore.

RESULTS

A Survey for Br. abortus *in the Tissues of* 5 *Pregnant Cows which were about to Abort after Experimental Infection with* Br. Abortus

The results shown in the Table clearly indicate that the fulminating infection was confined within the uterus and was almost entirely in the foetal cotyledons, fluids and chorion ; these tissues contained respectively 60–85 per cent, 1–25 per cent, and 2–8 per cent of the total organisms found in the mother and foetus. Viable counts done on these tissues indicated 30–50 per cent of the total organisms were viable.

Separation of Br. abortus *and its Products from Foetal Cotyledons and Fluids*

Filtered foetal fluids were collected in quantity (see above) from each cow. They contained the extracellular or easily removed surface products of *Br. abortus* grown *in vivo* ; there was no evidence of large scale lysis of *Br. abortus in vivo* from a comparison of the few serological precipitation lines (for details see later paper) produced by high concentrations of these fluids with the multiplicity of lines produced by extracts of *Br. abortus*.

The Table shows that the foetal cotyledon tissue and fluids contained 3–14 × 10^{13} *Br. abortus* ≡ approx. 1·4–6·6 g. of dry organisms (by comparison with the dry weight of known numbers of water washed *Br. abortus* grown *in vitro* ; 1 mg.≡ 2·1 × 10^{10} cells). Considering the intracellular habitat of the pathogen, the process summarized in the Fig. was efficient for the separation of *Br. abortus* from these sources. A good yield (40–80 per cent) of organisms was obtained and the great bulk of the contaminating host cellular debris was removed. Nevertheless the final product was not free from fine tissue debris ; although practically no debris could be observed microscopically in stained films of the final product, electron

microscopy revealed contaminating tissue debris which was similar to that seen on electron micrographs of the final product from normal cotyledon tissue which had been subjected to the same process as that described above for infected tissue. The following evidence indicated that the final preparation contained approx. 1 part organisms to 4 parts tissue debris. Five water washed preparations contained 4·7, 4·9, 5·1, 4·6 and 3·7 $\times 10^9$ organisms per mg. of dry preparation (60°, 0·1 mm. Hg., P_2O_5). A comparison of the average of these figures (4·6 $\times 10^9$

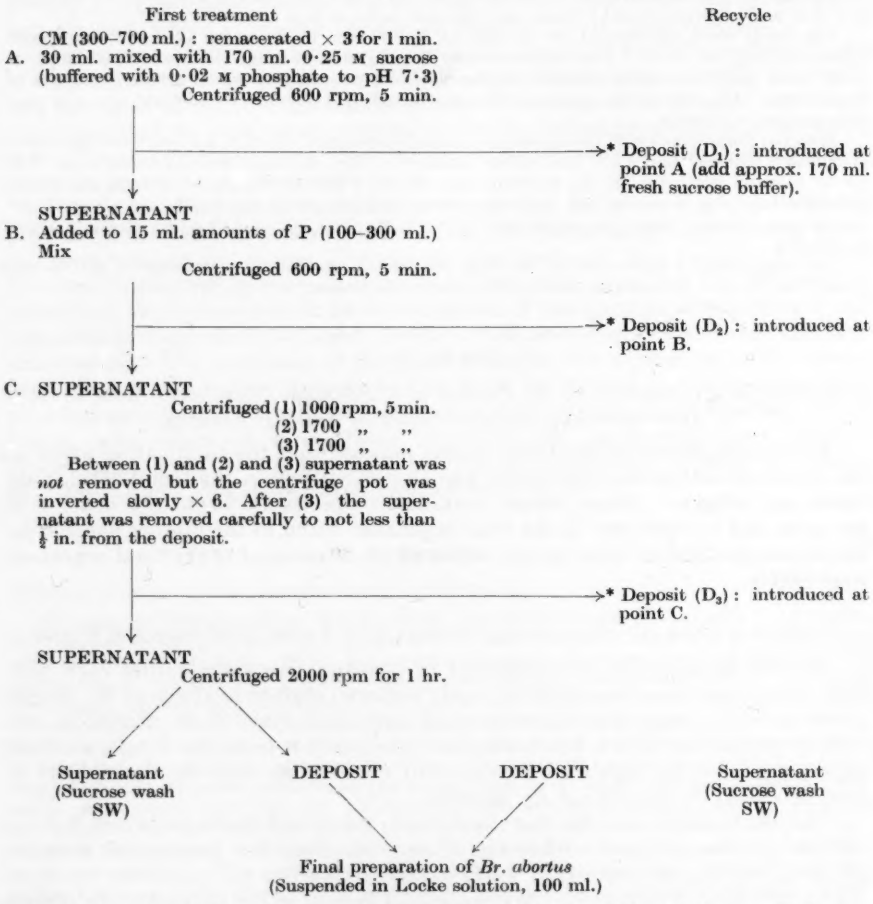
First treatment
Recycle
CM (300–700 ml.) : remacerated × 3 for 1 min.
A. 30 ml. mixed with 170 ml. 0·25 M sucrose (buffered with 0·02 M phosphate to pH 7·3)
Centrifuged 600 rpm, 5 min.
* Deposit (D1) : introduced at point A (add approx. 170 ml. fresh sucrose buffer).
SUPERNATANT
B. Added to 15 ml. amounts of P (100–300 ml.)
Mix
Centrifuged 600 rpm, 5 min.
* Deposit (D2) : introduced at point B.
C. SUPERNATANT
Centrifuged (1) 1000 rpm, 5 min.
(2) 1700 ,, ,,
(3) 1700 ,, ,,
Between (1) and (2) and (3) supernatant was not removed but the centrifuge pot was inverted slowly × 6. After (3) the supernatant was removed carefully to not less than ½ in. from the deposit.
* Deposit (D3) : introduced at point C.
SUPERNATANT
Centrifuged 2000 rpm for 1 hr.
Supernatant (Sucrose wash SW)
DEPOSIT
DEPOSIT
Supernatant (Sucrose wash SW)
Final preparation of Br. abortus
(Suspended in Locke solution, 100 ml.)

FIG.—Separation of *Br. abortus* from foetal cotyledon macerate (CM) and the pus (P) from foetal fluids.

All centrifugations were done at 0–3° in the refrigerated International Centrifuge Model PR1 (with 200–220 ml. pots) except the final centrifugation which was done in Model S.R.3 with not more than 400 ml. in each centrifuge pot (stainless steel cans with screw caps—for safety).

* The deposits obtained at these stages after the recycle were discarded.

TABLE.—*Distribution of* Br. abortus *in the Tissues of an Infected Pregnant Cow when Abortion was Imminent*

Tissue	Approx. total number ($\times 10^{12}$) of *Br. abortus* per tissue in cows				
	I	II	III	IV	V
Foetal tissue or fluid					
Cotyledon (placenta)	63	90	140	23	27
Allantoic fluid	0·4	0·9	3·7	7·9	1·2
Amniotic fluid	0·4	0·9	3·7	7·9	1·2
Chorion	5·6	2·1	9·9	0·6	3·3
Oedema fluid	0·9	—	1·4	—	—
Spleen, Kidney, Liver, Lung, Stomach contents, Brain	In no cow did the contents of *Br. abortus* in each of these foetal tissues exceed $0·1 \times 10^{12}$.				
Adult tissue					
Cotyledon (placenta)*	4·4	11	13	2·4	5·1
Uterine mucosa	—	—	3·0	2·4	0·4
Spleen, Kidney, Liver, Lung, Iliac lymph gland, Thoracic lymph gland, Udder, Muscle	In no cow did the contents of *Br. abortus* in each of these maternal tissues exceed $0·1 \times 10^{12}$.				

* It was impossible to separate completely all the foetal tissue from the maternal cotyledons.

per mg.) with the corresponding figure ($2·1 \times 10^{10}$ per mg.) for uncontaminated *Br. abortus* grown *in vitro* indicated the preparations obtained from infected tissue contained approx. 22 per cent w/w of *Br. abortus*. The degree of contamination with tissue debris could be reduced by further differential centrifugation but only at the expense of a much lower final yield of organisms.

Removal of Tissue Debris from Br. abortus *by Treatment with Trypsin*

The suspension of the final deposit of *Br. abortus* grown *in vivo* (100 ml.—see Fig.) was mixed with M/15 phosphate buffer at pH 8·0 (260 ml.) and heated to 37°. Freshly prepared trypsin solution (crystalline trypsin—Armour Laboratories—1 per cent w/v in M/15 phosphate buffer at pH 8·0 ; 40 ml., 37°) was added and the mixture was rotated at 37° for 1 hr. After centrifuging the mixture at 1750 *g.* for 30 min., the supernatant was removed leaving a deposit consisting of a brown layer above a grey layer. The brown layer contained tissue debris and hardly any *Br. abortus* ; it could be removed easily from the grey layer by a Pasteur pipette with a little M/15 phosphate buffer for washing. The grey layer which contained the organisms was washed once with M/15 phosphate buffer (pH8) and once with water; any slight amount of residual brown layer was removed after each washing (centrifuged at 1750 *g.* for 30 min.). The first supernatant and the subsequent washings were rendered sterile by filtration through Millipore and reserved for biological testing.

In several experiments, 60–80 per cent of the original *Br. abortus* was recovered in the final grey deposit which after washing ×3 with water contained 1·8–2·5 × 10^{10} organisms per mg. dry weight These figures agreed with the corresponding figure for uncontaminated *Br. abortus* grown *in vitro* (2·1 × 10^{10} ≡ 1 mg.) and indicated that the *Br. abortus* grown *in vivo* had been freed from contaminating tissue by the treatment with trypsin. This treatment may have removed some components of *Br. abortus* but it had no dramatic effect on the organism since adequate tests showed that the total count and the viability of a suspension of *Br. abortus* were not reduced by the treatment.

Extraction of Br. abortus *Grown* In Vivo

The final preparation of organisms or the trypsin treated final preparation was suspended in Locke solution (Ca^{++} omitted ; vol. to give 3 × 10^{11} organisms per ml.) and centrifuged at 0–2° for 30 min. at 10,000 rpm in the angle head of the M.S.E. centrifuge (Model " Angle 13 "). The sludge of organisms was treated twice in the Hughes Press (Hughes, 1951) which for reasons of safety was surrounded by cotton wool and placed in a plastic bag. Locke solution was added (vol. to give a suspension ≡ 3 × 10^{11} organisms per ml.) and the suspension centrifuged as described above. The supernatant was filtered through Millipore. The deposit (washed × 3) was suspended in Locke solution (vol. to give a suspension ≡ 3 × 10^{11} organisms per ml.) and sterilised either by heating to 60° for 1 hr. or by treatment with ultraviolet light (a thin layer, subjected to a Hanovia Bactericidal Ultraviolet unit, 0·25A 15 watts, at 8 cm. distance until sterility tests were satisfactory—approx. 2 hr.).

Hence in this work the following products were obtained from *Br. abortus* grown *in vivo*.; extracellular or easily removed surface products in allantoic and amniotic fluid, foetal oedema fluid, foetal cotyledon extract, and sucrose buffer (SW—see Fig.) used in separation of organisms ; cellular products—Hughes Press extracts and residues from the final preparation of *Br. abortus* before and after removing the tissue contamination with trypsin ; the latter treatment may have also removed or destroyed some bacterial products.

DISCUSSION AND SUMMARY

A survey of the numbers of *Br. abortus* in the tissues of 5 experimentally infected pregnant cows, when abortion was imminent, has shown that the fulminating infection was almost entirely confined to the foetal cotyledons, fluids and chorion which contained respectively 60–85 per cent, 1–25 per cent and 2–8 per cent of the total organisms (3–14 × 10^{13}) found in the mother and foetus. This survey underlined that a major unknown factor in the pathology of contagious abortion was the chemical basis for this predilection of *Br. abortus* for foetal tissue, and indicated that the heavily infected tissues were a rich source of *Br. abortus* grown *in vivo* which were needed for a study of the chemical basis of the intracellular growth and immunogenicity of *Br. abortus*.

By differential centrifugation of macerates of infected tissue and pus, 40–80 per cent of the *Br. abortus* (3–14 × 10^{13} ≡ approx. 1·4–6·6 g.) was concentrated in a preparation containing 1 part of bacteria to 4 parts of tissue debris. This tissue debris could be removed by treatment with trypsin. The organisms were

extracted in the Hughes Press either before or after removing the tissue debris. with trypsin.

These bacterial extracts and residues and the extracellular or easily removed surface products found in the infected foetal fluids and washing, will be examined in biological tests and extracted for important products responsible for pathogenicity and immunogenicity.

We are indebted to our colleague Dr. J. S. Paterson of Allington Farm, Porton for help with the collection of infected bovine tissues, and to our colleague Mr. R. B. FitzGeorge for work on treatment of organisms with trypsin.

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SURVEY OF PAPERS

KRISHNA MURTHY and SAHASRABUDHE investigated the cytology of pituitary cells in rats following bilateral adrenalectomy and autotransplantation of adrenal tissue into the spleen. During the stage of adrenal regeneration, hyperchromatic PAS-positive basophils appear in the pituitary. They regard these cells as probably responsible for the secretion of adrenocorticotrophic hormone. Further they found that these cells in the pituitary only appear in response to depletion of circulating corticoids, and the presence of target organs is essential for their appearance (p. 539).

JONES and ROITT have produced thyroiditis in rats by inoculating homologous or heterologous thyroid extract or thyroglobulin in Freund's adjuvants. Thyroiditis was consistently demonstrated 10 days after inoculation and reached its maximum severity about 6 days later. Regression of the lesions was apparent after this time. They discuss their findings in relation to the relevant human disease (p. 546).

COOPER and HOWARD injected a suspension of spleen cells from " A " mice intravenously into (C57BL × A) F1 hybrids. This caused increased reticulo-endothelial activity, as measured 14 days later by clearance of colloidal carbon from the blood. It also caused increased resistance to *D. pneumoniae* and *Salm. typhimurium* when these organisms were injected intravenously 12 days after the " A " cells (p. 558).

SELYE and GRASSO chemically induced cardiac necrosis and vascular occlusion in rats. Rats pre-treated with 9 α-fluorocortisol and Na_2HPO_4 developed areas of myocardial necrosis with thrombosis in the capillaries and medium sized veins of the heart following the intravenous administration of saccharated iron oxide. This is a selective site and is not accompanied by any apparent vascular damage within the heart (p. 564).

GREIG and ELSIE CORNELIUS have examined the effects of fat emulsions upon the fibrinolytic action of streptokinase upon normal human plasma clots. They found that high concentrations of various fats inhibited lysis, but yet could act as proactivator under certain conditions. Further they demonstrated that the emulsions have an affinity, which appears to be enhanced by incorporation of phosphatide, for a protein with properties like plasminogen which they can absorb from serum. Similar affinities on the part of platelets are demonstrated (p. 568).

LOUTIT and MICKLEM irradiated mice of strains CBA, A, and C3H which had previously been immunized against the iso-antigens of other strains. Injections of bone marrow and spleen cells from the immunizing strains, which protected control mice, failed to prevent radiation disease in immunized mice. This type of immunity could be passively transferred with serum where host and donor differed at the H-2 histocompatibility locus, but in the absence of this difference immune serum was ineffective (p. 577).

DAVIES and ANN HUTCHISON immunized mice by subcutaneous injection of ascites tumour cells of a heterologous strain. Ascitic fluid containing antibodies to the immunizing material was produced in the mice by intraperitoneal injection of isologous or non-specific tumour cells. In the presence of dextran and normal human serum this fluid agglutinated specifically mouse erythrocytes having the appropriate H2 histocompatibility antigens. The antibodies responsible could be specifically absorbed from the fluids by cells and by antigenic material in suspension or solution. They caused specific mixed agglutination of ascites tumour cells with erythrocytes, but failed to form a precipitation line with antigen in gel diffusion (p. 587).

JASMIN and GAREAU induced muscle lesions in rats by the s.c. injections of dimethylparaphenylenediamine hydrochloride. The heart and the gastrocnemius muscle were used for histological examination.

The rats were killed daily and the progression of the muscular lesions studied. The muscles initially showed swelling and homogenization of the fibres which subsequently underwent coagulation necrosis and fragmentation. These dead muscle fibres acted as foreign bodies and induced a histiocytic reaction.

Regeneration then occurred and this was complete by the 20th day with no fibrous or fatty tissue replacement except in the heart where the degenerate myocardial cells became fibrotic.

They discuss the similarity of these changes to those occurring in the primary myopathies (p. 592).

KIDSON has measured the lipid synthesis of human leucocytes from normal subjects and from cases of infective leucocytosis. He measured the total lipids, neutral lipids and phospholipids of the cells and plasma using a technique involving uptake of ^{14}C-labelled acetate. The results showed low rates of total lipid synthesis in infective leucocytosis, but the proportional synthesis of neutral lipids and phospholipids differed little from the normal. The lowest rates of synthesis occurred in the cases with the highest proportion of band neutrophils and synthesis rose to normal levels as the total count and the proportion of band leucocytes fell to normal (p. 597).

WALTER, JUDITH FRANK and IRWIN immunized rabbits with bovine serum albumin (BSA), an initial dose of 50 mg. in Freund's adjuvant into the toe pads, followed by 3 weekly intravenous doses of 10 mg. in saline.

Anaphylactic shock was caused by the intravenous injection of 50 mg. BSA. The lungs were observed *in vivo* during shock by the quartz rod method. The animals died within minutes or were killed within 30 min. Tissue sections from the lungs and other organs stained in various ways showed hyaline emboli particularly in the lungs, which stained like fibrin. It could not be proved that they were fibrin, but certainly they were not cellular. The authors opined that they were formed in the circulating blood (p. 603).

WALTER and JUDITH FRANK, using the same methods as in the preceding paper, found that the titre of precipitin to BSA in the blood of the sensitized rabbits before shock was unrelated to the number of emboli seen in lung sections after shock. This titre was related to the severity of the shock. The number of emboli seen in the lung *in vivo* was roughly related to that subsequently found in sections (p. 609).

FARTHING inoculated guinea-pigs with diphtheria toxoid and a dead *Bordetella pertussis* vaccine. He found that the serum antitoxin levels of these animals were much higher than in animals which received toxoid only (p. 614).

SPECTOR has investigated the role of changes in the water content of the brain following anoxic-ischaemic encephalopathy in rats. He found that cerebral oedema followed the procedure, commencing after about 4 hr. and reaching its maximum at about 12 hr. Some protection was afforded to the brain by giving 30 per cent urea i.v. after the anoxia or by lowering the environmental temperature during the anoxic period (p. 623).

SMITH, KEPPIE, PEARCE, FULLER and WILLIAMS have conducted a survey of the numbers of *Br. abortus* in the tissues of pregnant cows. They found that fulminating infection was largely confined to the foetal cotyledons, fluids and chorion. These results indicated that an unknown factor was the chemical basis for the predilection of the organism for foetal tissue. Methods of producing bacterial extracts and residues for further study are described (p. 631).